Characterisation of thiazide-sensitive pathways of sodium reabsorption in the kidney

A thesis by

Jodie Nadal

The thesis is submitted to the University of Nottingham for the degree of Doctor of Philosophy

School of Medicine, University of Nottingham





March 2019

The University of Nottingham

To Mark, Sarath, Neil and my parents

Publications and Published Abstracts

Jodie Nadal, Sarath K. Channavajjhala, Wenjing Jia, Jenny Clayton, Ian P. Hall and Mark Glover (2018) Clinical and Molecular Features of Thiazide-Induced Hyponatremia; Curr Hypertens Rep; 20 (31); 1-7

Jodie Nadal, Sarath K. Channavajjhala and Mark Glover. O-14: Investigation of The Relationship between Serum Potassium Concentration, 24h Dietary Potassium Intake and Expression of The Thiazide-Sensitive Sodium-Chloride Cotransporter in Patients with Hypertension; British and Irish Hypertension Society

Abstract

With increasing individuals developing hypertension and with no known cause, it is becoming ever more vital to research how our blood pressure is regulated throughout the body. The kidney is often a forgotten regulatory organ for blood pressure, however, it's biochemical role for increasing and decreasing pressure within the circulatory system is essential to maintain a healthy balance. This thesis aims to further characterise the functions of thiazide-sensitive sodium-chloride cotransporter located within the distal nephron of the kidney using urinary and cell culture exosomes. The co-transporter plays a key role in regulating blood pressure for genetic conditions like Gordon Syndrome and Gitelman Syndrome, providing the possibility to regulate blood pressure in essential hypertension.

As a source for protein and nucleic acids, the use of urinary exosomes is a novel non-invasive procedure. Urinary exosomes were extracted from urine and cell culture media allowing the collection of genetic and proteomic information from the kidney tubule cells. Specific biomarkers have been identified, in order to ensure the study of the area of interest for example the thiazide-sensitive sodium-chloride co-transporter (NCC) or ALG-2-interacting protein X (ALIX).

Validation protein work in chapter 3 was performed to determine the optimal conditions for exosomal fraction storage and quantify the exosomal fraction from the ultrafiltration extraction method, using NCC and phosphorylated NCC. It was identified that ALIX should be used as an exosomal marker over Aquaqporin-2 (AQP2). These validation methods were applied to the rest of the thesis and the communication experiments presented in Chapter 3. These communication experiments aimed to show exosomes as a communication vessel by transferring exosomal NCC into Proximal Kidney Tubule Cells (PKTC). Despite initial findings showing that exosomes can transfer NCC to PKTC, it was concluded further experiments were needed to confirm if NCC had transferred successfully and was physiologically functional.

Using ultra-filtration methods demonstrated in Chapter 3, exosomes were extracted from donated normonatraemic, non-thiazide patient urine to demonstrate the influence dietary electrolytes have on the expression and activation of NCC. In Chapter 4, 24hr electrolyte excretion was used as an alternative to electrolyte intake, which were then correlated to NCC and other relatable proteins. Decreasing levels of expression were seen in phosphorylated NCC when correlated with serum potassium producing a negative correlation, but the opposite was observed when serum potassium was compared to NCC_3 or $NCC_{1/2}$. In addition to serum potassium, 24hr urinary potassium did not correlate with NCC or phosphorylated NCC. In addition to potassium, other electrolytes e.g. chloride were correlated with NCC. It was identified that serum chloride negatively correlated with NCC_3, but not with phosphorylated NCC or $NCC_{1/2}$.

It has long been reported that exosomes hold Ribonucleic acid (RNA), which may be used as a form of communication between cells. However, extracting viable messenger RNA (mRNA) has proven difficult due to its instability outside of the exosomal environment. The studies presented in this thesis attempt various methods to extract the best quality and quantity of mRNA. The top four methods for extracting mRNA were followed up with Next Generation RNA Sequencing (performed by Oxford Genomics) in attempt to detect exosomal RNAs. Although the extracted exosomal RNA was poor quality, RNA sequencing was sensitive enough to report protein coding genes. Some of the RNAs detected by RNA sequencing were validated using Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR). The combination of RNA sequencing and qRT-PCR identified WNK Lysine Deficient Protein Kinase 1 (WNK1) and Cullin-3 (CUL3) from the WNK-SPAK/OSR1-NCC pathway.

RNA sequencing studies were continued to compare RNA expression between Acute and Convalescent Thiazide-Induced Hyponatremia (TIH) patients and control groups using the Seramir RNA extraction from Chapter 5's validation experiments. Additional RNase inhibitor was used to improve RNA quality. No known RNAs associated with TIH were identified despite using the hypothesis free method and the candidate approach method. It may be that despite

attempts to reduce degradation, the exosomal RNA suffered due to this factor, hence why no associated genes were identified. However, glucose-related proteins, *GYS2* and *SLC5A2,* were reported as differentially expressed between acute and convalescent TIH samples, suggesting that glucose channels may play a role in the development of TIH. Further pathway analysis, as well as genomic and proteomic analysis would help identify with these glucose-related proteins affect the pathophysiology of TIH patients.

Overall, the studies presented in this thesis provide further evidence towards thiazide-sensitive sodium-chloride cotransporter's relationship towards dietary electrolytes within a healthy human body, whilst also showing that exosomes can be used to characterise protein expression of the thiazide-sensitive sodium-chloride cotransporter and its regulatory proteins. The work carried out in this thesis has commented on the advantages and disadvantages of working with urinary exosomes, but it has also contributed to the growing knowledge of how these extracellular vesicles can be used to study the intracellular functions of cells without invasive biopsies.

Acknowledgements

I would like to thank Dr Mark Glover for all his guidance, support and the opportunity to complete this research throughout my time with his group.

My genuine thanks also go to Dr Sarath Channavajjhala for his patience, generosity in time and support for experimental and technical help. Thank you to all the collaborative projects completed together.

Thank you to the Medical Research Council (MRC), University of Nottingham and Dr Mark Glover for all the financial support given during the duration of my research.

I would also like to dedicate my thesis to my family and best friend:

- To my parents for their constant, never-ending support and always believing in me to achieve my greatest.
- To my brother for providing support all the way from Edinburgh.
- To my soon-to-be husband, Neil, for his daily positivity, humour and encouragement, so I could pursue my research.
- To my best friend Alex, who listened to my worries and doubts, in addition to proofreading my thesis.

And to my Grandad for being my scientific inspiration.

Contents

PUBLICATIONS AND PUBLISHED ABSTRACTS ..... **II**

ABSTRACT ..... **III**

ACKNOWLEDGEMENTS ..... **VI**

DECLARATION ..... **XVIII**

ABBREVIATIONS ..... **XIX**

1 INTRODUCTION **2**

1.1 GENERALISED AIM 2

1.2 HYPERTENSION 3

1.3 END ORGAN DAMAGE CAUSED BY HYPERTENSION 4

1.4 DIAGNOSING HYPERTENSION 5

1.5 RENAL SODIUM REABSORPTION 8

1.5.1 Thiazide-sensitive sodium-chloride cotransporter *9*

1.6 MENDELIAN DISORDERS OF RENAL SODIUM REABSORPTION 10

1.6.1 Bartter syndrome *10*

1.6.2 Gordon Syndrome *12*

1.6.3 Liddle Syndrome *13*

1.6.4 Hypertension Brachydactyly Syndrome *13*

1.6.5 Gitelman Syndrome *13*

1.7 GORDON SYNDROME AND WNK 14

1.7.1 WNK1 ..... *14*

1.7.2 WNK4 ..... *16*

1.8 WNK-OSR1/SPAK1 NCC PATHWAY 17

1.9 HORMONAL REGULATION 18

1.9.1 Angiotensin/Aldosterone *18*

1.9.2 Insulin *19*

1.10 UBIQUITINATION REGULATION 20

1.10.1 CUL3 ..... *20*

1.10.2 KLHL3 ..... *21*

1.11 REGULATORY EFFECTS OF ELECTROLYTES 22

1.11.1 Sodium *22*

1.11.2 Potassium *22*

1.11.3 Chloride *23*

1.11.4 Calcium *24*

1.12 THIAZIDE-INDUCED HYPONATREMIA 25

1.12.1 Current treatment of Hypertension *25*

1.12.2 ACE inhibitors ... *25*
1.12.3 Calcium-channel blockers ... *25*
1.12.4 Thiazide-like diuretics ... *26*
1.12.5 The Causes of Hyponatremia ... *28*
1.12.5.1 Thiazide-Induced Hyponatraemia ... 28
1.12.5.2 Syndrome of Inappropriate Antidiuretic Hormone ... 30
1.12.5.3 Heart failure ... 30
1.13 USING EXTRACELLULAR VESICLES TO STUDY RENAL PATHOLOGY ... 31
1.13.1 Microvesicles ... *32*
1.13.2 Apoptotic bodies ... *32*
1.13.3 Exosomes ... *32*
1.13.4 Urinary Exosomes ... *33*
1.13.5 Exosomes and Biomarkers ... *34*
1.13.5.1 Tamm-Horsfall protein ... 34
1.13.5.2 Aquaporin-2 ... 34
1.13.5.3 ALIX ... 36
1.13.6 Exosomal intercellular communication ... *37*
1.13.7 Exosomes use for understanding kidney disease ... *39*
1.14 AIMS, HYPOTHESIS AND OBJECTIVES ... 40
2 METHODS AND MATERIALS ... 42
2.1 CLINICAL RECRUITMENT ... 42
2.2 ANALYSIS OF BIOCHEMICAL ELECTROLYTES ... 42
2.3 URINARY EXOSOME EXTRACTION ... 44
2.3.1 Ultrafiltration ... *44*
2.3.2 Exoquick ... *44*
2.3.3 miRCURY ... *44*
2.3.4 exoEasy ... *45*
2.4 QUANTIFICATION OF URINARY EXOSOMAL PROTEINS BY WESTERN BLOTTING. ... 46
2.4.1 SDS-PAGE Electrophoresis ... *46*
2.4.2 Western blotting using the wet technique ... *48*
2.4.3 Western Blot Protein Detection by ECL Method ... *49*
2.5 RNA EXTRACTIONS ... 50
2.5.1 miRCURY ... *50*
2.5.2 ExoRNeasy ... *50*
2.5.3 SeraMir ... *51*
2.5.4 GenElute ... *51*
2.5.5 ZRNA isolation ... *51*
2.6 RNA AND PROTEIN VALIDATION ... 52
2.6.1 NanoDrop® ... *52*

2.6.2 Bicinchoninic Acid Assay (BCA) ... *52*
2.6.3 Ribogreen ... *53*
2.6.4 Bioanalyzer ... *53*
2.7 POLYMERASE CHAIN REACTION ... 54
2.7.1 Synthesis of cDNA ... *54*
2.7.2 Quantitative PCR using Taqman ... *56*
2.7.3 Analysis of qPCR using Taqman ... *57*
2.8 RNA SEQUENCING ... 58
2.8.1 RNA concentration ... *58*
2.8.2 Library quantification and sequencing of exosomal RNA ... *59*
2.8.3 Analysis of sequencing data ... *62*
2.8.3.1 Concatenation of Data ... 63
2.8.3.2 Adapter, Quality Trimming and FastQC Analysis ... 63
2.8.3.3 Tophat2 Alignment ... 67
2.8.3.4 Differential Gene Expression Analysis ... 67
2.8.3.5 Using Cuffnorm to determine gene expression ... 67
2.8.3.6 Command lines and codes ... 67
2.9 CELL CULTURE PREPARATION ... 70
2.9.1 General methods ... *70*
2.9.2 Transformation and Transfection of HEK-293 cells with a pcDNA5/FRT Empty Vector ... *71*
2.9.2.1 Transformation ... 71
2.9.2.2 Mini-prep and sequencing ... 72
2.9.2.3 Maxi-prep ... 72
2.9.2.4 Transfection ... 73
2.9.3 Collection of exosome rich media ... *74*
2.9.4 Harvesting cells for protein work ... *75*
2.9.5 Labelling Cells and Exosomes ... *75*
2.9.6 Imaging HEK and PKTC cells ... *76*
2.9.7 Exosomal NCC exchange to PKTC cells ... *76*
2.10 STATISTICAL ANALYSIS ... 77
2.10.1 Power calculation ... *77*
2.10.2 Tests of Normality ... *77*
2.10.3 Linear Regression ... *77*
2.10.4 Correlation Analysis ... *78*
2.10.5 One-way Anova ... *78*

3 VALIDATING THE USE OF URINARY EXOSOMES FOR WESTERN BLOTTING AND ITS ROLE IN EXTRACELLULAR COMMUNICATION 80

ABSTRACT 80

3.1 INTRODUCTION 82

3.2 OPTIMAL URINE FRACTIONS AND STORAGE CONDITIONS FOR EXOSOMES 85

3.2.1 Urine Fractions 85

3.2.2 Storage Conditions 85

3.2.3 The expression of phosphorylated NCC 88

3.2.4 Positive and Negative controls 89

3.2.5 AQP2: an alternative to ALIX? 90

3.3 THE ROLE OF EXTRACELLULAR VESICLES IN INTERCELLULAR COMMUNICATIONS BETWEEN HEK CELLS 91

3.3.1 HEK293 and related cell lines expression of NCC 91

3.3.1.1 NCC expression from HEK cell lines in 6-well plates 91

3.3.1.2 NCC expression from HEK cell lines in T75 flasks 92

3.3.2 NCC exosomal communication between HEK293 and Proximal Tubule Kidney Cells 96

3.4 DISCUSSION 100

3.4.1 Optimal urine fractionation to study human exosomes NCC and AQP2 100

3.4.2 Storage Conditions 100

3.4.3 Phosphorylated NCC 101

3.4.4 Positive and Negative Controls 101

3.4.5 Comparisons of exosomal markers 102

3.4.6 Exosomal communication between epithelial cells 102

4 SERUM AND DIETARY POTASSIUM EFFECTS ON EXPRESSION OF THIAZIDE-SENSITIVE SODIUM-CHLORIDE COTRANSPORTER WITHIN HUMAN URINARY EXOSOMES 106

ABSTRACT 106

4.1 INTRODUCTION 108

4.2 CHARACTERISTICS OF NORMONATRAEMIC HYPERTENSIVE PATIENTS 111

4.3 CORRELATION BETWEEN ELECTROLYTE, AND EXOSOMAL PROTEIN EXPRESSION 113

4.3.1 24hr urinary potassium excretion vs Serum potassium 114

4.3.2 NCC_3 115

4.3.3 $NCC_{1/2}$ 115

4.3.4 pNCC 118

4.3.4.1 pNCC S91 118

4.3.4.2 pNCC T55 119

4.3.4.3 pNCC T60 119

4.3.5 SPAK 123

4.3.6 ROMK 125

4.3.7 WNK4 125

4.4 DISCUSSION 127
4.4.1 Serum and Dietary Potassium/Sodium 127
4.4.2 Chloride's effect on NCC 129
4.4.3 Serum and dietary calcium and NCC 130
4.4.4 No WNK4 found in exosomes 131
4.5 SUMMARY 131

5 OPTIMISING THE EXTRACTION OF URINARY EXOSOMAL MRNA 134

ABSTRACT 134
5.1 INTRODUCTION 135
5.2 RESULTS 138
5.2.1 Nanodrop, Ribogreen and Bioanalyzer Validation 139
5.2.1.1 Nanodrop 139
5.2.1.2 Ribogreen 140
5.2.1.3 Bioanalyzer RNA 600 Pico Assay 141
5.2.2 Sequencing of exosomal RNA 144
5.2.3 Expression of SLC12A3 and related genes 146
5.2.3.1 Quality control Checks 146
5.2.3.2 Alignment to the Human Genome 149
5.2.4 Validating RNA-sequencing with qRT-PCR 153
5.2.5 RACK1 and TRPM6 expression using Taqman primers 153
5.2.5.1 Optimisation with control Fetal Kidney Total RNA 154
5.2.6 WNK1 and CUL3 are the highest expressed candidate genes within urinary exosomes . 155
5.2.6.1 CUL3 155
5.2.6.2 WNK1 157
5.2.6.3 Q-PCR comparison across extraction methods 159
5.3 DISCUSSION 161
5.3.1 Extraction methods 161
5.3.2 RNA-sequencing and qPCR 163

6 DIFFERENTIAL GENE EXPRESSION BETWEEN ACUTE AND CONVALESCENT THIAZIDE-INDUCED HYPONATRAEMIA PATIENTS 166

ABSTRACT 166
6.1 INTRODUCTION 167
6.2 RESULTS 170
6.2.1 RiboGreen validation 170
6.2.2 RNA-sequencing 173
6.2.3 Sequencing quality control 174
6.2.4 Alignment against the Human Genome 177
6.2.5 Differential expression of mRNA between Acute and Convalescent TIH patients 179

6.2.5.1 Differential expression with Fold Change 179
6.2.5.1.1 Acute TIH vs Convalescent TIH 182
6.2.5.1.2 Acute TIH vs C1 controls 185
6.2.5.1.3 Convalescent TIH vs C2 controls 188
6.2.5.2 Differential expression by directional fold change 191
6.2.5.3 Identifying genes based on population expression patterns 193
6.2.5.3.1 Heatmap of Acute TIH and Convalescent TIH samples 193
6.2.5.3.2 Heatmap of Acute TIH and C1 control samples 196
6.2.5.3.3 Heatmap of Convalescent TIH samples and C2 control samples 198
6.2.5.4 Differentially expressed genes representative of the sample population 200
6.2.6 Candidate mRNA approach 203
6.3 DISCUSSION 205
6.3.1 The use of the Hypothesis Free Approach 205
6.3.2 Candidate gene approach 206
6.3.3 Glucose-related proteins 207
6.3.4 Summary 208
7 GENERALISED DISCUSSION AND CONCLUSIONS 210
7.1 HYPOTHESIS AND AIMS 210
7.2 OVERALL FINDINGS 211
7.2.1 Urinary exosomes can be used for measuring mRNA and protein expression 211
7.2.2 Potassium's effect on pNCC expression and regulation of NCC 212
7.3 LIMITATIONS 213
7.4 PROPOSED FUTURE WORK 215
7.4.1 Identifying urinary exosomal protein expression by quantitative methods 215
7.4.2 Confirmation of exosomal NCC communication by confocal microscopy 217
7.4.3 Exosomal pathway analysis between Acute and Convalescent TIH patients 217
7.5 FINAL CONCLUSIONS 218
8 REFERENCES 219
9 APPENDIX 234
9.1 CDNA NANODROP PICTOGRAPHS 234
9.2 PUBLISHED PAPER 238
9.3 ETHICAL APPROVAL 243
9.3.1 Thiazide-Induced Hyponatraemia Ethical Protocol 243
9.3.2 Thiazide-Induced Hyponatremia Patient Information Sheet 273
9.3.3 Thiazide-Induced Hyponatremia Regulatory Sheet 277
9.3.4 Gordon Syndrome Ethical Protocol 280
9.3.5 Gordon Syndrome Patient Information Sheet 300
9.3.6 Gordon Syndrome Regulatory Sheet 306

Table of Figures

FIGURE 1-1 STRUCTURE AND SODIUM REABSORPTION SEGMENTS OF THE NEPHRON. 8
FIGURE 1-2 ILLUSTRATION OF A NEPHRON, SHOWING MENDELIAN DISORDERS OF HYPERTENSION AND HYPOTENSION AND THEIR ASSOCIATED GENE PRODUCTS 11
FIGURE 1-3 WNK-SPAK1/OSR1-NCC CASCADE 17
FIGURE 1-4 HORMONAL REGULATION OF NCC]. 19
FIGURE 1-5 NICE GUIDELINE CG127 ON HYPERTENSION DRUG TREATMENT. 27
FIGURE 1-6 PRODUCTION OF EXOSOMES AND MICROVESICLES 31
FIGURE 1-7 THE REGULATION OF AQP2 BY THE CAMP CASCADE. 35
FIGURE 1-8 FUNCTION OF ALIX IN PRODUCTION OF EXOSOMES 36
FIGURE 1-9 MICROVESICLES AND EXOSOMAL COMMUNICATION WITH A RECIPIENT CELL. 38
FIGURE 2-1 QPCR CYCLING PROCEDURES 57
FIGURE 2-2 THE PURIFICATION OF RNA SAMPLE USING MAGNETIC BEADS. 58
FIGURE 2-3 SUMMARY OF LIBRARY PREP OF RNA SAMPLES BEFORE SEQUENCING 60
FIGURE 2-4 SUMMARY OF RNA SEQUENCING AFTER LIBRARY PREP.. 61
FIGURE 2-5 FLOWCHART OF CUFFLINKS PACKAGE V2.2.0 62
FIGURE 3-1 STRUCTURAL DIFFERENCES BETWEEN THE EXTRACELLULAR VESICLES 83
FIGURE 3-2 EXPRESSION OF AQP2 AND NCC IN URINE FRACTIONS. 86
FIGURE 3-3 EXPRESSION OF AQP2 AND NCC IN URINE EXOSOMES STORED AT VARIOUS TEMPERATURES 87
FIGURE 3-4 COMPARISON OF EXPRESSION OF TOTAL NCC AND PNCC IN URINE EXOSOMES 88
FIGURE 3-5 COMPARING NCC EXPRESSION BETWEEN GITELMAN AND GORDON SYNDROME SAMPLES. 89
FIGURE 3-6 COMPARISON BETWEEN ALIX AND AQP2 EXPRESSION. 90
FIGURE 3-7 B-ACTIN EXPRESSION IN HEK, HEK-NCC AND HEK-EV EXOSOMES AND CELL PELLETS. EXOSOMAL SUPERNATANT AND CELLS WERE HARVESTED FROM T75 FLASKS. 92
FIGURE 3-8 NCC AND FLAG-NCC EXPRESSION IN HEK EXOSOMES AND CELL PELLETS 94
FIGURE 3-9 NCC AND FLAG-NCC EXPRESSION IN HEK EXOSOMES AND CELL PELLETS GROWN IN T75 FLASKS 95
FIGURE 3-10 PKTC CELLS AT 60-70% CONFLUENCY UNDER BRIGHTFIELD 96
FIGURE 3-11 PKTC CELLS LABELLED WITH QTRACKER® LABELLING KIT 97
FIGURE 3-12 EXPRESSION OF NCC AND FLAG-NCC IN PKTC CELLS WITH AND WITHOUT HEK EXOSOMES (N=3). 98
FIGURE 4-1 MECHANISMS OF REGULATING NCC. 109
FIGURE 4-2 CORRELATION BETWEEN 24HR K^+ EXCRETION AND SERUM K^+ CONCENTRATIONY. 114
FIGURE 4-3 LINEAR CORRELATIONS OF NCC_3 BETWEEN SERUM K^+ CONCENTRATION, SERUM Na^+ CONCENTRATION, SERUM Cl^- CONCENTRATION, SERUM Ca^{2+} CONCENTRATION, 24HR Na^+ EXCRETION, 24HR K^+ EXCRETION, AND 24HR Cl^- EXCRETION. EACH INDIVIDUAL POINT REPRESENTS A PATIENT. ABU = ARBITRARY UNITS. 116
FIGURE 4-4 LINEAR CORRELATION BETWEEN $NCC_{1/2}$ AND SERUM K^+ CONCENTRATION, SERUM Na^+ CONCENTRATION, SERUM Cl^- CONCENTRATION, SERUM Ca^{2+} CONCENTRATION, 24HR Na^+ EXCRETION, 24HR K^+ EXCRETION, AND 24HR Cl^- EXCRETION. EACH INDIVIDUAL POINT REPRESENTS A PATIENT. ABU = ARBITRARY UNITS. 117

Figure 4-5 Linear correlation between pNCC S91 and Serum K^+ concentration, Serum Na^+ concentration, Serum Cl^- concentration, Serum $Ca2^+$ concentration, 24hr Na^+ excretion, 24hr K^+ excretion, and 24hr Cl^- excretion. Each individual point represents a patient. Abu = Arbitrary units. 120

Figure 4-6 Linear correlation between pNCC T55 and Serum K^+ concentration, Serum Na^+ concentration, Serum Cl^- concentration, Serum $Ca2^+$ concentration, 24hr Na^+ excretion, 24hr K^+ excretion, and 24hr Cl^- excretion. Each individual point represents a patient. Abu = Arbitrary units. 121

Figure 4-7 Linear correlation between pNCC T60 and Serum K^+ concentration, Serum Na^+ concentration, Serum Cl^- concentration, Serum Ca^{2+} concentration, 24hr Na^+ excretion, 24hr K^+ excretion, and 24hr Cl^- excretion. Each individual point represents a patient. Abu = Arbitrary units. 122

Figure 4-8 Linear correlation between SPAK and Serum K^+ concentration, Serum Na^+ concentration, Serum Cl^- concentration, Serum Ca^{2+} concentration, 24hr Na^+ excretion, 24hr K^+ excretion, and 24hr Cl^- excretion. Each individual point represents a patient. Abu = Arbitrary units. 124

Figure 4-9 Linear correlation between ROMK and Serum K+ concentration, Serum Na+ concentration, Serum Cl- concentration, Serum Ca2+ concentration, 24hr Na+ excretion, 24hr K+ excretion, and 24hr Cl- excretion. Each individual point represents a patient. Abu = Arbitrary units. 126

Figure 5-1 Nanodrop pictograph for Seramir extraction method 139

Figure 5-2 Average concentration (with SEM) of each extraction method samples using Ribogreen assays. 140

Figure 5-3 Average concentration (with SEM) of Extraction Method samples from the Bioanalyzer RNA 6000 Pico Assay. 141

Figure 5-4 Bar chart to show the comparison of the concentration of RNA before and after using AMPure beads. 144

Figure 5-5 Bioanalyzer Gel images showing the distribution of mRNA lengths. 145

Figure 5-6 Serial Dilution of RACK1 and TRPM6 Amplification plots and Ct values of Total Fetal Kidney RNA. The PCR protocol ran for 40 cycles..... 154

Figure 5-7 qRT-PCR Ct values of CUL3 expression. 156

Figure 5-8 qRT-PCR Ct values of WNK1 expression. 158

Figure 5-9 Comparison of Sample 7 across the top 4 extraction methods. 160

Figure 6-1 AQP2, NCC and PGT protein expression in TIH patients and controls.. 168

Figure 6-2 Top 20 gene comparisons between Acute, convalescent and controls by fold change.. 180

Figure 6-3 All differential expressed genes compared against Acute, convalescent and controls by fold change..... 181

Figure 6-4 Heatmap of the top 20 differentially expression genes between acute and convalescent TIH samples..... 183

Figure 6-5 Heatmap of the top 20 differentially expression genes between acute TIH and control samples.... 187

Figure 6-6 Heatmap of the top 20 differentially expression genes between convalescent TIH and control samples..... 190

FIGURE 6-7 COMBINED DIFFERENTIAL EXPRESSED RNAS AFTER FOLD CHANGE AND DIRECTIONAL FOLD CHANGE ANALYSIS. 192
FIGURE 6-8 HEATMAP OF TOP DIFFERENTIALLY EXPRESSED RNAS IN INDIVIDUAL SAMPLES OF ACUTE TIH AND CONVALESCENT TIH PATIENTS. 195
FIGURE 6-9 HEATMAP OF TOP DIFFERENTIALLY EXPRESSED RNAS IN INDIVIDUAL SAMPLES OF ACUTE TIH PATIENTS AND C1 CONTROLS. 197
FIGURE 6-10 HEATMAP OF TOP DIFFERENTIALLY EXPRESSED RNAS IN INDIVIDUAL SAMPLES OF CONVALESCENT TIH PATIENTS AND C2 CONTROLS 199
FIGURE 9-1 E1-E10 CDNA NANODROP PICTOGRAPHS 234
FIGURE 9-2 EM1-EM5 CDNA NANODROP PICTOGRAPH 234
FIGURE 9-3 EP1-EP10 CDNA NANODROP PICTOGRAPH 234
FIGURE 9-4 G1-G10 CDNA NANODROP PICTOGRAPH 235
FIGURE 9-5 GP1-GP10 CDNA NANODROP PICTOGRAPH 235
FIGURE 9-6 M1-M10 CDNA NANODROP PICTOGRAPH 235
FIGURE 9-7 MG1-MG5 CDNA NANODROP PICTOGRAPH 236
FIGURE 9-8 MP1-MP10 CDNA NANODROP PICTOGRAPH 236
FIGURE 9-9 SP1-SP10 CDNA NANODROP PICTOGRAPH 236
FIGURE 9-10 Z1-Z10 CDNA NANODROP PICTOGRAPH 237

Table of Tables

TABLE 1-1 SECONDARY CAUSES OF HYPERTENSION. 7
TABLE 2-1 EQUIPMENT USED TO MEASURE ELECTROLYTES, VITAMINS AND HORMONES OF TIH PATIENTS 43
TABLE 2-2 VOLUMES OF COMPONENTS FOR COMPOSITION OF RESOLVING GELS 46
TABLE 2-3 VOLUMES OF COMPONENTS FOR COMPOSITION OF STACKING GELS. 46
TABLE 2-4 LIST OF PRIMARY AND SECONDARY ANTIBODIES USED 48
TABLE 2-5 EXPOSURE TIMES FOR MEMBRANES. 49
TABLE 2-6 RNA STANDARDS 53
TABLE 2-7 REAGENTS USED TO MAKE RNA/PRIMER MASTER MIX PER SAMPLE. 55
TABLE 2-8 REAGENTS USED FOR 2X MASTER MIX PER SAMPLE. 55
TABLE 2-9 PRE-DESIGNED PRIMERS USED WITH TAQMAN QPCR 56
TABLE 2-10 REAGENTS REQUIRED PER SAMPLE PER WELL FOR QPCR. 56
TABLE 2-11 DETAILS OF EACH FASTQC OUTPUT. 64
TABLE 2-12 LIST OF COMMAND, THEIR FUNCTIONS AND COMMAND LINES. 68
TABLE 2-13 CODES USED TO WITHIN COMMAND LINES.. 69
TABLE 2-14 REAGENTS TO MAKE COMPLETE MEDIA 70
TABLE 2-15 REAGENTS FOR USE OF BAMH1 WITH THE PCDNA5. 73
TABLE 2-16 LIST OF REAGENTS USED TO MAKE TRANSFECTION MIX IN 1 WELL FROM A 6-WELL PLATE 74
TABLE 2-17 REAGENTS FOR PROTEIN LYSIS BUFFER USED TO HARVEST CELLS 75
TABLE 3-1 CELL COUNTS OF THE 3 HEK CELL LINES IN 4 T75 FLASKS. 92
TABLE 4-1 DESCRIPTIVE DATA OF CHARACTERISTICS OF NORMONATRAEMIC, NON-THIAZIDE VOLUNTEER PATIENTS. 112
TABLE 4-2 PERCENTAGES OF ANTI-HYPERTENSIVE DRUGS TAKEN. 113
TABLE 5-1 ADVANTAGES AND DISADVANTAGES OF EXTRACTION METHODS FOR RNA 136
TABLE 5-2 SAMPLE NAMES AND THEIR RNA EXTRACTION PROCESS 138
TABLE 5-3 NANODROP, RIBOGREEN AND BIOANALYZER CONCENTRATIONS OF RNA EXTRACTION METHOD. 142
TABLE 5-4 CONTINUED… 143
TABLE 5-5 SAMPLE S2 (SERAMIR RNA EXTRACTION METHOD) FASTQC REPORT.. 147
TABLE 5-6 ALIGNMENT DATA FROM SAMPLES SEQUENCED. 149
TABLE 5-7 LIST OF CANDIDATE GENES WITH ESTABLISHED FUNCTION IN DISTAL NEPHRON SODIUM OR WATER TRAFFICKING AND THEIR FPKM NUMBERS IN EACH SAMPLE. 150
TABLE 6-1 RIBOGREEN RESULTS SHOWING MRNA CONCENTRATION OF TIH AND GS SAMPLESS. 172
TABLE 6-2 SAMPLE 233 FASTQC REPORT. 174
TABLE 6-3 ALIGNMENT PERCENTAGE AND READ DEPTH OF ALL SAMPLES SEQUENCED. 178
TABLE 6-4 TOP 20 GENES EXPRESSED BETWEEN ACUTE TIH SAMPLES AND CONVALESCENT TIH WHEN ORDERED BY FOLD CHANGE. 182
TABLE 6-5 TOP 20 GENES EXPRESSED BETWEEN ACUTE TIH SAMPLES AND C1 CONTROLS WHEN ORDERED BY FOLD CHANGE. 185

TABLE 6-6 TOP 20 GENES EXPRESSED BETWEEN CONVALESCENT TIH AND C2 CONTROLS WHEN ORDERED BY FOLD CHANGE. THE TOP 20 GENES ARE LISTED BY FOLD CHANGE..... 188

TABLE 6-7 ADDITIONAL RNAS IDENTIFIED BY DIRECTIONAL FOLD CHANGE ANALYSIS BETWEEN ACUTE TIH SAMPLES VS C1 CONTROL SAMPLES..... 191

TABLE 6-8 ADDITIONAL RNAS IDENTIFIED BY DIRECTIONAL FOLD CHANGE ANALYSIS BETWEEN CONVALESCENT TIH SAMPLES VS C2 CONTROL SAMPLES..... 191

TABLE 6-9 DIFFERENTIALLY EXPRESSED RNA'S BETWEEN ACUTE TIH AND CONVALESCENT TIH SAMPLES..... 200

TABLE 6-10 DIFFERENTIALLY EXPRESSED RNA'S BETWEEN ACUTE TIH AND C1 CONTROL SAMPLES..... 201

TABLE 6-11 DIFFERENTIALLY EXPRESSED RNA'S BETWEEN CONVALESCENT TIH AND C2 CONTROL SAMPLES..... 202

TABLE 6-12 CANDIDATE GENES DIFFERENTIALLY EXPRESSED BETWEEN ACUTE TIH SAMPLES AND CONVALESCENT TIH SAMPLES..... 203

TABLE 6-13 CANDIDATE GENES DIFFERENTIALLY EXPRESSED BETWEEN CONVALESCENT TIH SAMPLES AND C2 CONTROL SAMPLES..... 203

Declaration

The work described in this dissertation is completely my own unless specifically stated in the text. This dissertation has not been previously submitted for another degree, diploma, or any other qualification at any university of the institution.

Any guidance or technical and experimental advice has been fully appreciated and acknowledged.

This thesis contains less than 100,000 words.

Miss Jodie R Nadal, BSc (Hons), MSc (Hons)

Abbreviations

ABPM	Ambulatory Blood Pressure Monitoring
ANOVA	One-Way Analysis of Variance
ACE	Angiotensin-Converting-Enzyme
ADH	AntiDiuretic Hormone
ALIX	ALG-2-interacting protein X
ALG-2	Apoptosis-Linked Gene 2
Ang II	Angiotensin II
AQP2	Aquaporin 2
AVP	Vasopressin
BP	Blood Pressure
BSA	Bovine Serum Albumin
BCA	Bicinchoninic Acid Assay
BS	Bartter Syndrome
Ca^{2+}	Calcium
CASR	CAlcium Sensing Receptors
cDNA	Complementary DNA
Cl^-	Chloride
CD	Collecting Duct
CNT	CoNnecting Tubule
Ct	Cycle Threshold

CVD	CardioVascular Disease
CUL3	Cullin-3
DASH	Dietary Approaches to Stop Hypertension
DCT	Distal Convoluted Tubule
DNA	Deoxyribonucleic acid
eGFR	Estimated Glomerular Filtration Rate
ENaC	Epithelial sodium (Na) Channel
ECL	Enhanced ChemiLuminescence
FHHt	Familial Hyperkalaemia and Hypertension (also known as Gordon Syndrome)
FPKM	Fragments Per Kilobase of transcript per Million mapped
GS	Gitelman Syndrome
HBPM	Home Blood Pressure Monitoring
K^+	Potassium
KLHL3	Kelch-Like Family Member 3
KO	Knock-Out
KS-WNK	Kidney-specific WNK Lysine deficient protein kinase
LncRNA	Long Non-Coding RNA
L-WNK	Long WNK Lysine deficient protein kinase
MAPK	Mitogen-Activated Protein Kinase
Mg^{2+}	Magnesium

mRNA	messenger RiboNucleic Acid
Na^+	Sodium
NCC	Sodium-chloride cotransporter
Nedd4-2	NEural precursor cell expressed Developmentally Down-regulated 4-like
NGS	Next Generation Sequencing
NICE	The National Institute for Health and Care Excellence
NKCC1/2	Sodium-Potassium-Chloride cotransporter 1/2
OSR1	Oxidative Stress-Responsive kinase-1
P60	Proline60
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
QC	Quality Control
PCT	Proximal Convoluted Tubule
PGT	ProstaGlandin Transporter
PHA	PseudoHypoAldosteronism
PP4	Protein Phosphatase 4
PSA	Prostate-Specific Antigen
PTH	ParaThyroid Hormone
PVDF	PolyVinylidene DiFluoride
RAS	Renin-Angiotensin System
RBX1/2	RING finger protein 1/2

RT	Reverse Transcriptase
RIN	RNA Integrity
RNA	Ribonuclease Acid
RNA-seq	RNA-sequencing
ROMK	Renal Outer Medullary Potassium channel
RTe	Room Temperature
S91	Serine91
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate–PolyAcrylamide Gel Electrophoresis
SGK1	Serum/Glucocorticoid-regulated Kinase 1
SIADH	Syndrome of Inappropriate Antidiuretic Hormone
SLC12A1/2/3	Solute Carrier Family 12 Member 1/2/3
SPAK	STE20(sterile 20)/SPS1-related proline/Alanine-rich Kinase
T55	Theronine55
TAL	Thick Ascending Loop of Henle
TBS-T	TBS+0.1% v/v Tween
THP	Tamm-Horsfall Protein
UF	Ultrafiltration
WNK1	WNK Lysine deficient protein kinase 1
WNK4	WNK Lysine deficient protein kinase 4

Chapter 1: Introduction

1 Introduction

1.1 Generalised Aim

With increasing numbers of individuals developing hypertension and with approximately 30% having 'essential hypertension' i.e. high blood pressure without an obvious underlying medical cause, it is becoming ever more vital to research how our blood pressure is regulated. The kidney is often a forgotten regulatory organ for blood pressure, overshadowed by the circulatory system. However, the kidney plays an important biochemical role in maintaining the healthy electrolyte balance within the body. Abnormal sodium and chloride reabsorption caused by kidney dysfunction can cause deviations of blood pressure. This thesis aims to further characterise the functions of the thiazide-sensitive sodium-chloride cotransporter within exosomes. This transporter is located within the distal nephron of the kidney and moves sodium and chloride from the distal tubule into the epithelial cells. The sodium and chloride continue into the blood via other transporters on the basal side of the cell. It plays a key role in blood pressure control for genetic conditions like Gordon syndrome and Gitelman syndrome, which illustrates its potential ability to regulate blood pressure in essential hypertension. It is also thought to play a role in the electrolyte disorder Thiazide-Induced Hyponatraemia. The experiments presented in this thesis aim to show the importance of using urinary exosomes to study renal pathology, as well as how they can be used to measure gene and protein expression specifically of the sodium-chloride cotransporter.

1.2 Hypertension

Since 2600BC, when it was known as the "hard pulse disease", hypertension has been a growing challenge, affecting individuals worldwide [1, 2]. It is the most common reversible risk factor for CardioVascular Disease (CVD), with a third of the UK and world adult population currently suffering from hypertension [3-5]. Yet surprisingly, hypertension is usually at least partly reversible through regular medication or changes in lifestyle. Currently, treatment for hypertension and its vascular complications cost the NHS billions of pounds every year, therefore it is important to find more cost-effective ways to manage hypertension [6, 7].

Although the cause of hypertension in any given individual is often unknown, there are certain sections of the population that are at significant risk of developing the condition. These include [8]:

- People over the age of 65
- A high amount of salt in their diet
- African or Caribbean origin
- Lacking regular exercise
- People with a BMI >25
- Those with a diet poor in fruit and vegetables

Hypertension can be divided into two types: primary (essential) or secondary hypertension. Primary hypertension is the most common form of the condition and is defined by not being linked to any other medical problems such as renal failure or hyperaldosteronism. Instead, it is a complex product of interactions between the environment and genetics [1, 9, 10].

A major environmental contributor to hypertension is sodium chloride (NaCl), through either excess dietary consumption or renal retention. Blood pressure is determined, at least in part, by the balance between the amount of salt intake and its excretion into the urine [6]. Today on average more than 12g/day is consumed by the general population, and restricting total salt intake to 5g/day could prevent 1.25 million deaths from strokes and 3 million from cardiovascular disease per year across the world [3].

Secondary Hypertension is caused by an underlying disease resulting in the development of high blood pressure e.g. hormonal disorders, vascular abnormalities, renal disorders, or single gene mutations (monogenic or Mendelian hypertension) (see Table 1-1).

Monogenic forms of hypertension are caused by a single genetic mutation, and with the single exception of Hypertension Brachydactyly (which affects the blood vessels' contractivity), share excessive retention of sodium salt in the Distal Convoluted Tubule (DCT) as a common mechanism of pathogenesis [6].

Mendelian disorders of hypertension, as well as other drug-induced conditions e.g. thiazide-induced hyponatraemia, present unusual and important opportunities to further our understanding of sodium regulation within the kidney, and so inform our understanding of blood pressure regulation in humans.

1.3 End organ damage caused by Hypertension

Although most cases of essential hypertension are treatable, controlling blood pressure with medication can be extremely challenging for a minority. Unfortunately, the lack of control of blood pressure leads to severe complications, which can affect morbidity and mortality [11]. According to WHO in 2013, patients with uncontrolled hypertension are likely to develop hypertensive-related diseases within their lifetime [11]. Worldwide, at least 45% of hypertensive patients will die from heart disease, whilst 51% of patients are in danger of suffering from stroke [11]. Although heart disease and stroke are the most common, high blood pressure also affects other organs leading to further complications if it remains untreated, including kidney failure and blindness [11].

1.4 Diagnosing Hypertension

Patients that are suffering from high blood pressure rarely present with symptoms. However, there have been some reports of headaches, blurred vision, and shortness in breath [12]. A patient's blood pressure is usually taken during a GP check-up, where hypertension may be identified.

Blood pressure is routinely measured using sphygmomanometers. The manual method uses a cuff and bladder. The cuff wraps around the arm, whilst the bladder is used to increase the air and pressure within the cuff. Once at maximum pressure, the air is released whilst using a stethoscope to detect the Korotkoff sounds to determine systolic and diastolic blood pressure. More recently, blood pressure has been measured with automated devices allowing for increased accuracy and objectively removing user bias. However, healthcare professionals mist ensure the device is properly validated, maintained and recalibrated regularly [4].

Alternatively, Ambulatory Blood Pressure Monitoring (ABPM), 24-hour monitoring, may be undertaken using an electronic device, which allows repeated and a more reliable estimate of a patient' blood pressure throughout the day [13].

Systolic pressure measures the pressure in the arteries when the heart contracts (systole), whilst the diastolic pressure measures the pressure in the arteries between heartbeats i.e. heart is resting and refilling with blood (diastole).

$$\text{Hypertension} = \frac{\text{Systolic pressure (>140mmHg)}}{\text{Diastolic pressure (>90mmHg)}}$$

A patient is diagnosed as hypertensive when the systolic pressure is higher than 140mmHg and the diastolic pressure is higher than 90mmHg. As the normal range for healthy blood pressure is anywhere between 140/90mmHg and 90/60mmHg, low blood pressure (hypotension) is considered to be lower than 90/60mmHg or a pressure which causes symptoms of hypotension such as light-headedness [11].

According to the criteria adopted by NICE, when a diagnosis of hypertension is likely, a measurement should be taken in both arms [4]. If blood pressure is above the recommended measurement, a second reading should be taken during the current appointment [4]. If measurements differ, another reading should be repeated, recording the lower of the three readings [4].

If blood pressure does read above 140mmHg/90mmHg, healthcare professionals should offer ABPM or Home Blood Pressure Monitoring (HBPM) in order to confirm a hypertensive diagnosis [4]. For HBPM, hypertensive readings are measured at 135mmHg/85mmHg [4]

In some cases, patients may present with white coat hypertension. Although it affects about 15% of patients, this form of high blood pressure only presents itself when in a medical environment, caused by anxiety [13]. If "white-coat" hypertension is suspected, a patient may be given a device to measure blood pressure whilst at home using HBPM [4].

Table 1-1 Secondary causes of hypertension.
The secondary causes of Hypertension are considerably more common than monogenic/Mendelian Hypertension, with vascular hypertension as the most common of the secondary causes. Monogenic Hypertension is useful as a clinical example for studying the causes of conditions.

System	Disease	Mechanism
Vascular	Coarctation of the aorta/Renal artery stenosis	Hyperreninemia from reduced blood flow to the in kidneys
Endocrine	Hyperaldosteronism e.g. Conn's syndrome	Hyperplasia or adenoma genes: Genes: CYP11B1/B2, KCNJ5
	Phaeochromocytoma	Adrenal medulla causing episodic hyperadrenalism, tachycardia and peripheral vasoconstriction (10%=malignant/90%=benign) Genes: VHL syndrome, RET in MEN 2 syndrome, NF2, SDH genes
	Cushing syndrome	Obesity, some mineralocorticoid effect, and increased vascular tone from hypercortsolaemia
	Acromegaly	Possible plasma volume expansion
Intra-renal	Renal parenchymal disease	Reduced nephron number with the inability to excrete Na^+ and water
Pregnancy	Pre-eclampsia	Defective angiogenesis causes an ischemic placenta which leads to increased vasoconstrictor (e.g. TXA2) and decreased vasodilator (e.g. PGI_2) in maternal circulation
Drugs	Sympathomimetics MAO inhibitors	Increased catecholamine-mediated HR and TPR
	Combined OCP	? fluid/Na retention by kidney ? increased hepatic angiotensinogen synthesis
	Liquorice, Carbenoxolone	Inhibition of 11βHSD type 1 leading to endogenous glucocorticoid mimicking mineralocorticoid excess

1.5 Renal sodium reabsorption

Once filtered, urine enters approximately 1 million nephrons within the kidney's cortex and medulla where many electrolytes and molecules are reabsorbed by proteins known as transporters on the apical membrane of epithelial cells [14]. Amongst the electrolytes and molecules, sodium is the principal extracellular cation and is absorbed throughout the nephron (Figure 1-1) [15]. From the glomerulus, sodium reabsorption begins in the proximal tubule (PCT) where approximately 65% of the total sodium is reabsorbed. This action causes a driving force for the reabsorption of HCO_3^-, amino acids and glucose further along the nephron.

The Thick Ascending Loop of Henle (TAL) reabsorbs 25% of sodium by the sodium-potassium-chloride cotransporter (NKCC1 and NKCC2).

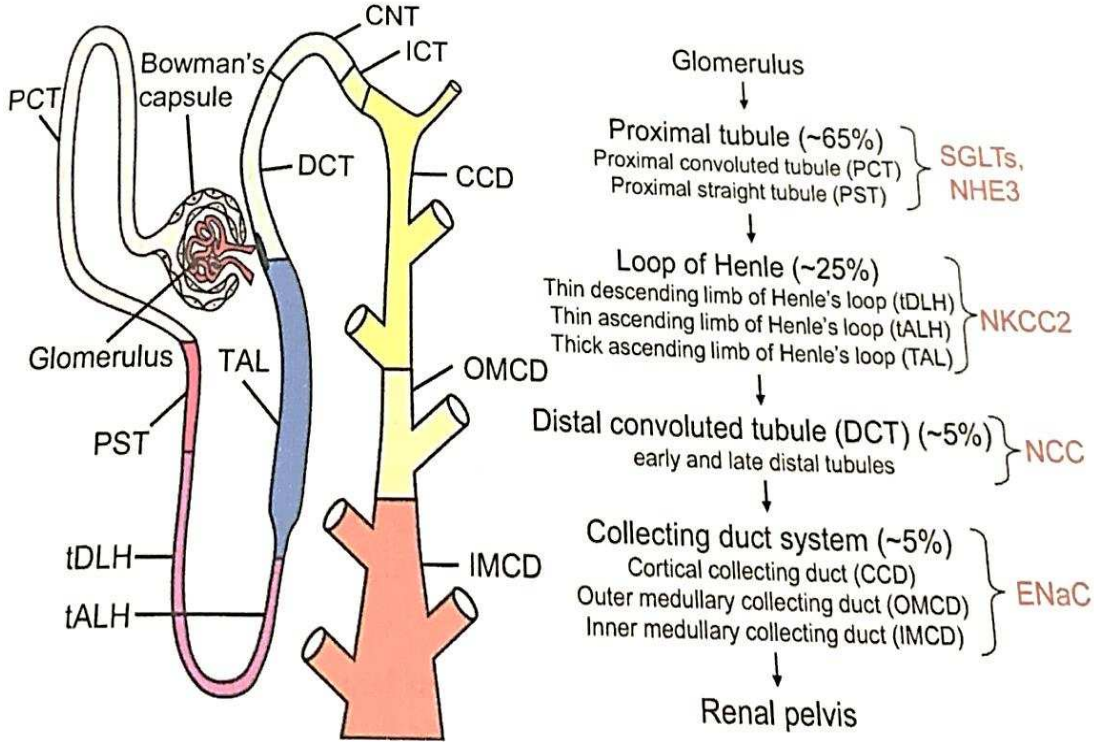


Figure 1-1 Structure and sodium reabsorption segments of the nephron.
The sodium reabsorption starts from the Bowman's capsule after glomerular filtration, with each section of the nephron expressing a different cotransporter, as well as reabsorbing smaller percentages of sodium [15].

Within the DCT, 5% of the sodium is reabsorbed into the blood. However, unlike the PCT, which reabsorbs more, the DCT is highly regulated, allowing more or less sodium to enter the blood depending on the physiological needs [15]. This fluctuation can change blood pressure, leading to hypertension or hypotension [16].

Finally, sodium reabsorption occurs in the collecting duct (CD) where 1-5% is reabsorbed into the blood. Sodium is transported through the epithelial cells by Amiloride-sensitive Epithelial sodium (Na^+) channel (ENaC) [15].

1.5.1 Thiazide-sensitive sodium-chloride cotransporter

Members of the major cotransporter families: *SLC12,* including *SLC12A3* (thiazide-sensitive sodium-chloride cotransporter or NCC) *SLC12A1* (NKCC2) and *SLC12A2* (NKCC1) (sensitive to loop diuretics), are responsible for reabsorbing sodium and other electrolytes from the nephron into the blood [14].

NCC is encoded by the *SLC12A3* gene. This is a 55kb gene and encodes 26 exons [17]. This is transcribed and translated into the NCC protein with an amino acid length between 1002 and 1029 long and a molecular weight of 140-175kDa [17, 18]. The protein forms a tertiary structure including a central hydrophobic domain with 12 putative transmembrane spanning regions, a hydrophilic region in the transmembrane domains 7-8, and also contains 2 glycosylation sites [17]. NCC's structure consists of an N-terminal serine-threonine rich domain and a Sterile20 STE20/SPS1-related proline/alanine-rich kinase / Oxidative Stress-Responsive Kinase-1 (SPAK/OSR1) binding domain, both of which are important in the regulation of the cotransporter by WNK and SPAK/OSR1 [19].

There are 3 isoforms of NCC present in the human kidney [20]. $NCC_{1\text{-}2}$ differs from NCC_3 by 9 amino acids [20]. The only difference between NCC_1 and NCC_2 is one amino acid, therefore it is virtually impossible to distinguish between these two isoforms by raising NCC isoform-specific antibodies [20]. One is known as NCC_3, which is the only isoform analysed in the majority of studies to date [20]. However, NCC_1 and NCC_2 make up 44% of the total NCC

expressed in the apical membrane of the DCT, yet little is known regarding the differential function and regulation these isoforms [20].

NCC is the only thiazide-sensitive sodium-chloride cotransporter expressed in the DCT, however, it has been questioned whether other cotransporters are also sensitive to thiazides. Using a rat model, Leveil *et* al. suggested that up to 50% of the sodium reabsorption in the CD might also be thiazide sensitive. These thiazide-sensitive channels in the CD may consist of a Na^+ driven Cl^- /HCO_3^- exchanger and Na^+-independent anion exchanger (pendrin), which together may demonstrate an "NCC-like" activity [21]. However, this finding has not been replicated in other species, and so at present, it is reasonable to assume that in humans NCC is the only thiazide sensitive pathway of sodium reabsorption in the DCT [21].

1.6 Mendelian disorders of renal sodium reabsorption

Although clinically rare, there are several well-characterised Mendelian disorders of hypertension, which have informed our understanding of renal sodium handling and so the regulation of human blood pressure, as illustrated in Figure 1-2.

1.6.1 Bartter syndrome

The most proximal of the rare salt-wasting tubulopathies is Bartter Syndrome (BS), found in the TAL [14]. Unlike other monogenic salt regulation disorders, there are several types (1-5), each with mutations in different genes, which affect individuals from infancy. Each type is slightly different, owing to the different mutated genes [22]. BS types 1 & 2 are caused by mutations in NKCC2 and the Renal Outer Medullary Potassium channel (ROMK), respectively, with symptoms of polyhydramnios, polyuria, and nephrocalcinosis. Chloride voltage-Gated Channel Kb (CLCNKB) is mutated in BS type 3 showing symptoms similar to BS types 1&2, as well as volume contraction and muscle weakness [23]. Symptoms of BS type 4, including sensorineural deafness, are caused by a mutation in the gene *Barttin*. Finally, BS type 5 produces hypocalcaemia and suppressed parathyroid hormone by

mutations in the gene, encoding CAlcium sensing receptors (CASR) [22]. In addition to mutations in CASR, Melanoma-Associated Antigen D2 (MAGED2) has also been linked to the causations of antenatal Bartter syndrome 5. From an initial study, MAGED2 shows to affect NCC and NKCC2 expression, which can be fatal to fetal development [24].

The autosomal recessive Bartter syndrome is characterised by hypotension, chronic hypokalemia, metabolic alkalosis, and hyperreninemic hyperaldosteronism. Patients show symptoms of cramps and can develop nephrocalcinosis, growth retardation, tetanic crises and fatigue [14].

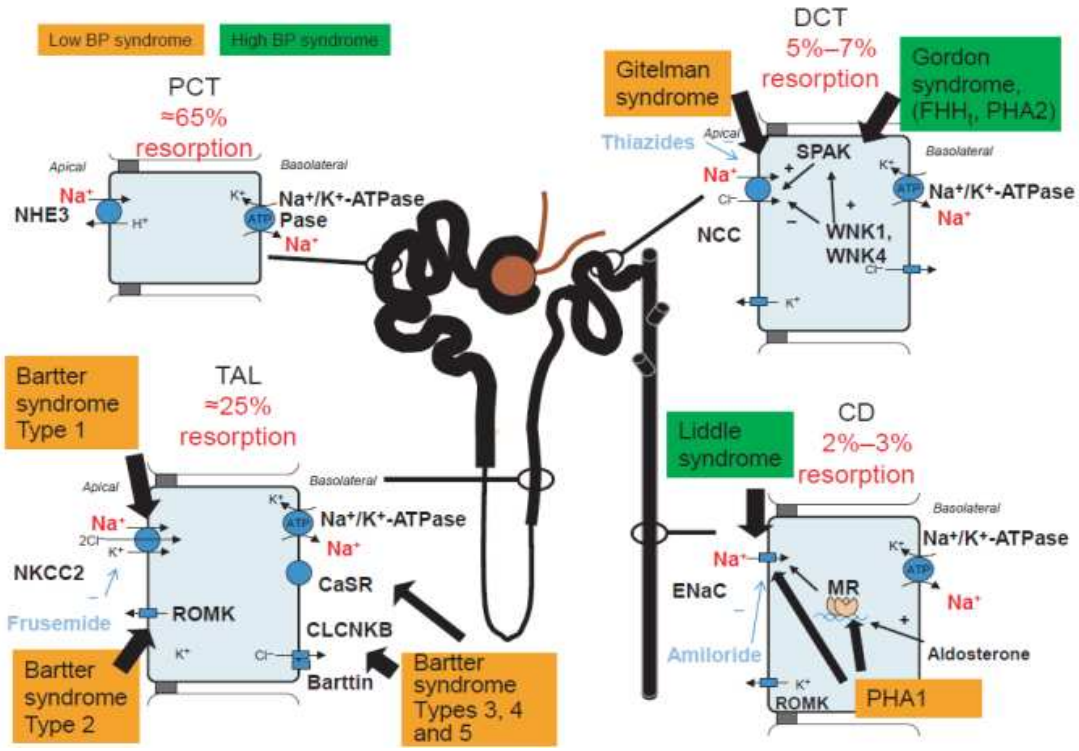


Figure 1-2 Illustration of a nephron, showing Mendelian disorders of hypertension and hypotension and their associated gene products. *In the PCT, there are no known genetic disorders, but it is responsible for the majority of reabsorption of sodium. The TAL reabsorbs 25% of filtered salt and is the site affected by the causes of Bartter syndromes. Mutations that cause Gordon and Gitelman syndrome are located in the DCT, which reabsorbs 5-7% of sodium. The CD is the final section of nephron which mutations within in this section cause Liddle syndrome and Pseudohypoaldosteronism type I (PHAI) [6].*

1.6.2 Gordon Syndrome

Gordon syndrome (Familial Hyperkalaemia and Hypertension (FHHt) or Pseudohypoaldosteronism type 2) is a Mendelian cause of hypertension which can be caused by at least four genetically distinct syndromes which result in excess reabsorption of sodium and chloride via the thiazide-sensitive NCC pathway [9]. The first report was made of an isolated case (an adopted 15-year-old boy) by Paver and Pauline in 1964 [25]. Subsequently, Richard Gordon described many more cases and undertook detailed physiological studies. It became clear that although isolated sporadic cases did occur, the condition tended to cluster in families and therefore was likely to be genetic in origin [26, 27]. To date, more than 150 pedigrees have been studied and reported [28]. Although the principal features of the physiology of the disease have been well documented, largely as a result of Gordon's work, the disease is not fully understood at a molecular level [18, 26].

The physical features of FHHt are thought to be related to childhood metabolic acidosis and include short stature, hypoplastic incisor teeth and muscle weakness [10]. FHHt is characterised by childhood hyperkalaemia, early adulthood salt-sensitive hypertension, low renin and metabolic acidosis, with normal glomerular filtration rate [9, 28]. FHHt is caused by mutations within *WNK1*, *WNK4*, *CUL3,* and *KLHL3* genes. The variant proteins produced by these mutations increase NCC expression substantially and lead to excessive NCC-mediated sodium reabsorption from DCT.

FHHt is treated with thiazide diuretics and a restricted salt diet. If diagnosed and treated promptly in infancy, most treated individuals can live a normal life with normal blood pressure and biochemical electrolytes. However, low bone mineral density does not seem to be prevented by early treatment with thiazides [14, 27].

1.6.3 Liddle Syndrome

Within the CoNnecting Tubule (CNT) and CD, ENaC is accountable for the remaining 2-3% of sodium reabsorption. ENaC is made up of three subunits: α, β and γ, of which either of the latter two subunits are mutated in an autosomal dominant Mendelian disorder known as Liddle syndrome. Mutations in the β and γ unit lead to the loss of the cytoplasmic tails preventing degradation of the cotransporter via Nedd4-2 and the Clathrin-coated pits pathway. The resulting accumulation (overexpression) of excess sodium reabsorption leads to the characteristic features of Liddle syndrome: hypertension and hypokalemic alkalosis associated with suppressed renin and aldosterone concentration [6]. This disorder responds well to the ENaC inhibitors Amiloride or Triamterene.

1.6.4 Hypertension Brachydactyly Syndrome

Hypertension Brachydactyly Syndrome is an autosomal dominant Mendelian form of hypertension, causing short stature as well as other mild dysmorphic features. However, unlike Liddle and FHHt, Hypertension Brachydactyly Syndrome is not a salt-sensitive condition and individuals with this condition do not display suppression of renin. However, their high blood pressure, beginning in childhood, continues to rise throughout their life resulting in premature death from stroke often before the age of 50 [29, 30].

Recently, the gene responsible for Hypertension Brachydactyly Syndrome was found to be Phosphodiesterase 3A and this is thought to cause hypertension by affecting arteriole blood vessels to increase peripheral resistance [29, 31].

1.6.5 Gitelman Syndrome

Unlike the diseases described above, patients with GS develop hypotension, hypokalemia, and metabolic alkalosis due to the mutations found in *SLC12A3* leading to decreased expression of NCC. More than 100 mutations have been reported to cause changes to the folding of the NCC and increased degradation due to the enhanced recognition by chaperone proteins. This autosomal recessive renal tubule disorder produces various symptoms, including growth retardation, cramps, fatigue, and chronic kidney disease.

1.7 Gordon Syndrome and WNK

With-No Lysine (k)-kinases (WNK) are a family of four genes that code for serine/threonine enzymes, which regulate NCC, with *WNK1* and *WNK4* as the most significant to the WNK-SPAK/OSR1 NCC pathway [26, 32, 33].

WNK kinases share a similar structure, including an N-terminal serine-threonine kinase domain, a SPAK-OSR1 binding domain and several coiled-coil domains [19]. Coiled-coil domains allow WNK homo-oligomers and hetero-oligomers to form, leading to autophosphorylation of the kinases in the presence of chloride [19].

The WNK-SPAK-NCC cascade has been of great interest to many research groups over the years, owing to its importance in regulating human blood pressure as demonstrated by FHHt [34]. Because of the many regulators and stimulators in this cascade, it makes it a very appealing target for novel drugs in principle, although the problems of physiological redundancy in regulators of epithelial transport and the challenge of finding a renal-specific target present substantial issues in developing such novel therapies [26].

1.7.1 WNK1

WNK1 is a major regulator in reabsorbing electrolytes and is predominantly expressed in the intracytoplasmic or basolateral membrane of epithelial cells in the DCT [35, 36].

WNK1 has 3 promoters: two are present before and within exon 1, whilst the other promoter lies in intron 4 [36]. The outcome of these different locations of promoters is to produce two different WNK1 isoforms: L-WNK1 and KS-WNK1. L-WNK1 is the full-length protein, whilst due to its promoter location in intron 4, KS-WNK1 is missing the kinase domain and hence much shorter than L-WNK1 [36].

Up until recently, L-WNK1 was thought to be solely involved in the regulation of electrolyte reabsorption. But Hadchouel and Verouti have both reported that WNK1 is critical to the embryonic development and proved this using WNK $^{-/-}$ KO mice, which led to embryonic lethality [36, 37]. Verouti went further to explain that the embryonic lethality concerned cardiovascular

defects, proving that WNK1 has more than just a role of regulation in kidney reabsorption [37]. However, Hadchouel concluded that it was difficult to confirm the function of L-WNK1, due to the potent expression of KS-WNK1 [36].

L-WNK1 acts as a powerful activator of NCC by inhibiting WNK4. However, L-WNK1 is regulated by its isoform, KS-WNK1, which by binding and blocking L-WNK1 from functioning thereby allows WNK4 to continue inhibiting NCC [17, 34]. In addition to KS-WNK1, chloride also greatly affects the activity of WNK1 by binding to a chloride binding domain and inhibiting autophosphorylation of WNK1 [28].

As with many components of biological pathways, WNK1 is regulated by internal and external modulators. Aside from KS-WNK1, the regulation of WNK1 is regularly debated, with some groups suggesting that hormones such as Angiotensin II and Aldosterone exert the biggest effect, whilst others propose electrolyte levels and the cell's general metabolic health dictate the activity of the kinase.

Of all the four genotypes of FHHt, *WNK1*^{FHHt} produces the least severe phenotype. The most common mutation is the lack of exon 11 and 12 [34]. This deletion also decreases ROMK activity, which may reduce the renal secretion of potassium and contributes to hyperkalaemia [17].

Various attempts have been made to find a link between *WNK1* and essential hypertension. Liu *et* al. reported rs880054, rs956868, rs12828016 variants (located in Intron 10, Exon 13 and Exon 21, respectively) in *WNK1*, but when the experiment was repeated by another group, it was found to be inconsistent [38]. This inconsistency might be due to the use of different populations of patients with essential hypertension.

To date, definite proof that variants within *WNK1* contribute to the development of essential hypertension has not been found.

1.7.2 WNK4

Unlike WNK1, there is only one isoform of WNK4. Although WNK4 is primarily expressed within the tight junctions of DCT and the cytoplasm of the CD, it has been shown that expression is also found in the colon, pancreas and sweat ducts [36, 39].

WNK4 has a variety of roles that affect the expression of NCC, as well as other electrolyte transporters. Its primary role is to inhibit NCC expression directly at the apical membrane, but the protein can also activate NCC through the phosphorylation of SPAK1/OSR1. In addition to these primary roles, WNK4 inhibits ENaC and ROMK and increases the expression of NKCC1/2 and paracellular chloride transport [9, 34].

Regulation of WNK4 is thought to be by insulin, angiotensin II and through ubiquitination from the Cul3-based E3 RING-type ubiquitin ligase complex. It is believed that angiotensin II reverses the inhibition effects of WNK4 on NCC [34].

The molecular mechanism by which WNK4 regulated NCC is still not completely understood. Sohara *et* al. and Yang *et* al. both agree that the inhibitory activity of the protein is dependent on the carboxyl-terminal [28, 36]. However, Wilson *et* al. argue that the inhibitory activity of WNK4 is found within the kinase domain. The missense mutations found in FHHt WNK4 proteins reverse the direct inhibitory effect of the protein, allowing dysregulation of the cotransporters [17, 33].

1.8 WNK-OSR1/SPAK1 NCC pathway

At least 12 interacting proteins are centred in the WNK cascade [40]. However, the immediate regulators of NCC and therefore blood pressure are SPAK and OSR1, which are activated in turn through phosphorylation by the WNK kinases. Both of these proteins are vital to regulating salt/fluid secretion, whilst also protecting cells from osmotic damage.

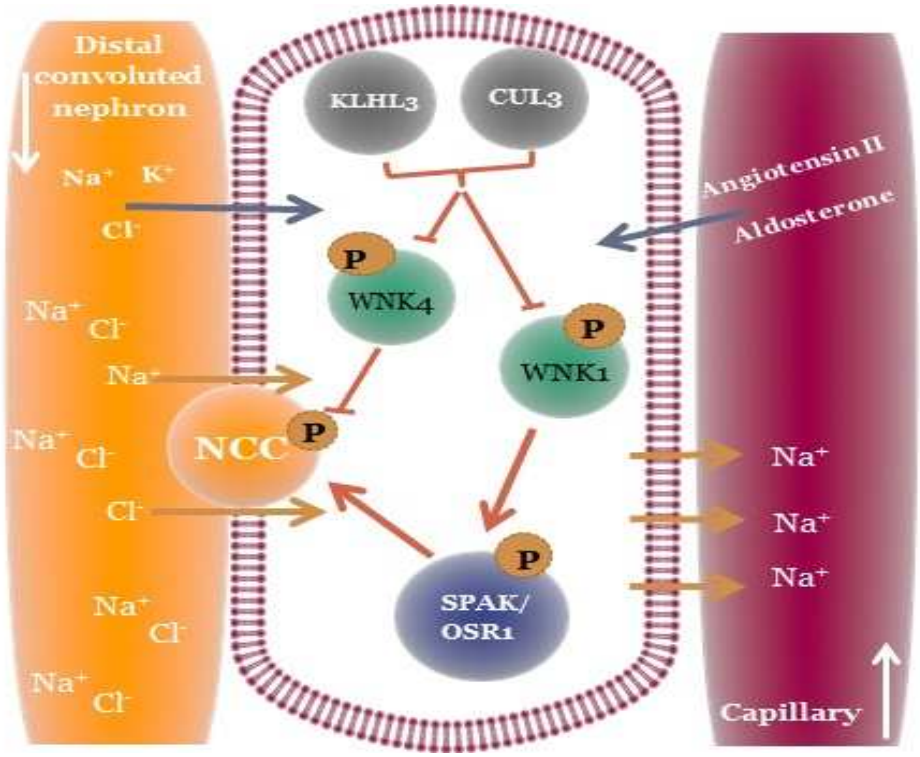


Figure 1-3 WNK-SPAK1/OSR1-NCC cascade. *The WNK kinases are modulated by several factors: Na^+, Cl^- and K^+ from the nephron and Angiotensin II and aldosterone from the blood. The presence of these factors can change whether WNK kinases autophosphorylate and hence activate the cascade, by phosphorylating SPAK1/OSR1, which progresses to phosphorylating NCC [16, 28]*

Using $SPAK^{-/-}$ KO-mice, Verouti showed a significant decrease in the phosphorylation and expression of NCC and NKCC2 when SPAK and OSR1 were absent [16]. This suggests that NCC and related proteins are regulated by SPAK and OSR1 by phosphorylating N-terminal threonines of NCC [41]. A decreased response to thiazide was also observed, suggesting the loss of NCC transporter function [16].

Further knock-in mice studies have also confirmed NCC's activation through SPAK and OSR1, as well as detailing SPAK and OSR1's activation and functionality through WNK kinases (WNK1 and WNK4) [42]. Rafiqi *et*, al. showed that SPAK and WNK play a vital role in the regulation of NCC and blood pressure, using SPAK knock-in mice. SPAK knock-in mice ($SPAK^{243A/243A}$) were not activated by WNK kinases, leading to reduced phosphorylation of NCC and hypotensive characteristics resembling Gitelman syndrome, [42].

1.9 Hormonal regulation

Increasing evidence shows that specific hormones are involved in blood pressure regulation due to the hormone effects of expression and regulation of NCC. Figure 1-4 shows hormone receptors implicated in the regulation of NCC through molecular cascades e.g. WNK-SPAK/OSR1-NCC and RasGRP/ERK1/2 [43].

1.9.1 Angiotensin/Aldosterone

The Renin-Angiotensin System (RAS) produces the angiotensin II and aldosterone, both of which play vital roles in electrolyte reabsorption and excretion within the kidney. By binding to the Mineralocorticoid Receptor (MR), aldosterone causes the downstream activation of NCC through the Nedd4-2 by activating transcription of the SGK1 gene [43]. Angiotensin II is a potent activator of NCC through $Angiotensin_1$ (AT_1) activating the WNK4-SPAK/OSR1-NCC pathway (Figure 1-4) [43].

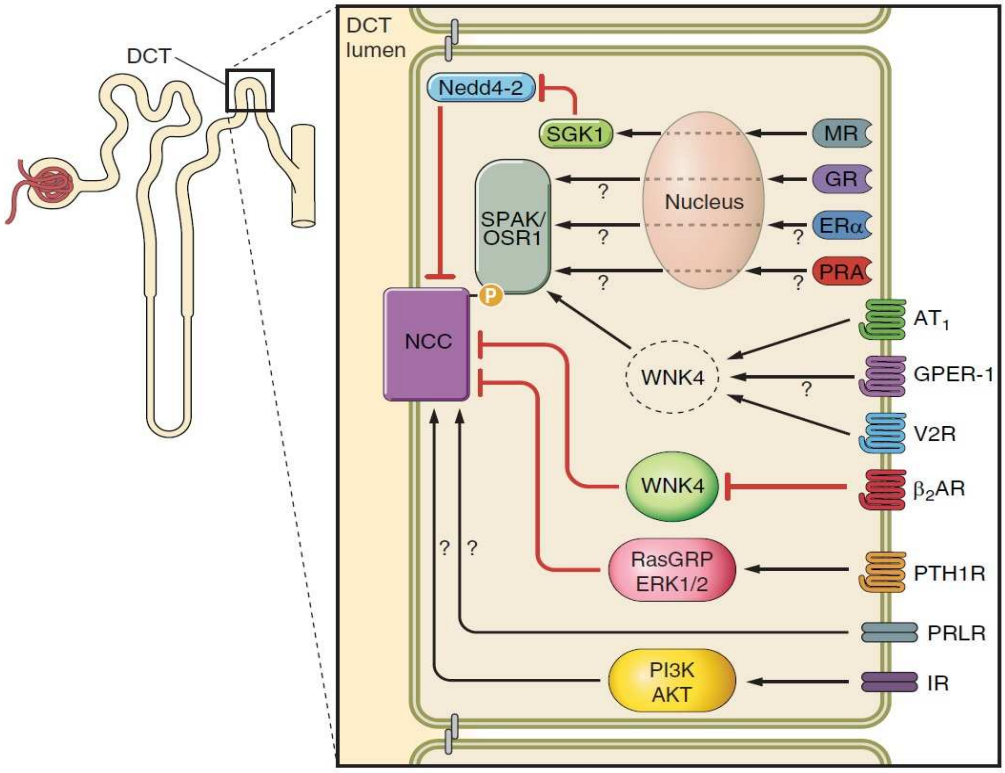


Figure 1-4 Hormonal regulation of NCC. *Various hormones affect the expression of NCC in many ways. Some directly affect gene expression e.g. mineralocorticoid, but other hormones regulate through indirect routes by activating upstream proteins e.g. Angiotensin (AT_1). GR=Glucocorticoid, ERα=oestrogen receptor, PRA=progesterone receptor, GPER-1=G-protein oestrogen receptor, V2R=vasopressin receptor, β_2AR=Adrenergic receptor, PTH1R=Parathyroid hormone receptor, PRLR=Prolactin receptor, IR=Insulin receptor [43].*

1.9.2 Insulin

Insulin is a powerful activator of NCC, by causing the phosphorylation of three key sites within the N-terminus of NCC: T55, T60 and S91 [44]. The PI3K/Akt cascade is a major signalling pathway for regulating NCC via the insulin receptor (Figure 1-4), but the involvement of the WNK4-SPAK pathway to activate NCC remains unclear [44-46].

Insulin plays an important role in promoting growth, which requires salt and water, therefore NCC's activation by the insulin receptor facilitates insulin's role [44]. Also, this activation helps the kidney retain an increased amount of salt immediately after eating a meal, as well as causing the intake of glucose into cells [44].

1.10 Ubiquitination regulation

Regulation of the WNK-OSR1/SPAK1 NCC pathway is partly through the Cul3-based E3 RING-type ubiquitin ligase complex which lies upstream of WNK proteins [40]. Recently, the discovery of specific mutations concerning Kelch-like 3 (KLHL3) and Cullin-3 (CUL3) in FHHt has changed the understanding of how the syndrome can be inherited. Mutations in *KLHL3* and *CUL3* were identified in 63% of pedigrees with FHHt, without mutations in WNK1 or WNK4 [40]. Mouse models of *CUL3* mutation have lower body weight and body length, which is thought to result from metabolic acidosis. This knowledge has been applied to children, with the result that several studies have shown that with thiazide treatment from childhood, the growth retardation in FHHt can be prevented or reversed, resulting in an increase in growth rate towards the normal range [40, 47].

Mutations in the genes coding for *KLHL3* and *CUL3*, lead to dysregulation of the ubiquitination complex causing over-expression of NCC and other cotransporters at the apical membrane [40].

1.10.1 CUL3

CUL3 is part of Cullin-RING ligase which binds to small RING finger proteins (RBX1/2), making CUL3 the catalytic core of the complex [48]. The heterozygous dominant mutations of CUL3 in FHHt cause severe symptoms in childhood, as well as increased blood pressure [48]. Schumacher *et* al. reported the findings of a mutation in CUL3, which explained the role CUL3 played in the regulation of WNK1/4 in further detail [48]. The exon 9 mutation led to the deletion of 56 amino acids and prevented the ubiquitination of WNK kinases by preventing the formation of Cul3-based E3 RING-type ubiquitin ligase [48]. However, it did not affect the ability to bind to the RING domain, which hydrolyses E2-UB.

1.10.2 KLHL3

KLHL3 is a key protein which is critical to the CUL3 ubiquitination complex, as it targets and makes the link between WNK kinases and CUL3-RBX1 [26, 48]. Different FHHt-causing KLHL3 mutations display different modes of inheritance: for example, missense mutations are autosomal dominant, whereas premature terminations, frameshift and splice site mutations are autosomal recessive [49]. The mutations found in FHHt affect the binding of WNK1 and WNK4 leading to loss of interaction between WNK and KLHL3 [49].

As well as regulating the expression of NCC, KLHL3 also plays a role in the expression of ROMK in the CD through inhibiting WNK4. However in FHHt, mutations in KLHL3 cause a reduction in the ubiquitination of WNK4, therefore WNK4 is overexpressed and leads to increased inhibition of ROMK, and increased activity in NCC [49]. This results in hyperkalaemia and metabolic acidosis.

1.11 Regulatory Effects of electrolytes

Electrolytes from the glomerular filtrate play an important role in the regulation of basolateral ion-transporters. Too little or too much electrolyte can cause pathological changes. The most crucial electrolytes are sodium, potassium, chloride, and calcium.

1.11.1 Sodium

Normal physiological sodium concentration in human serum is 135-145mmol/L. Less than 135mmol/L is termed hyponatraemia, whilst above 145mmol/L is termed hypernatremia [50].

Dietary sodium excess is common in the western world's diet and linked to the increase in cardiovascular diseases. It is still unclear to how sodium causes increased blood pressure. Some studies have shown increasing the concentration of dietary sodium leads to an increased expression of NCC and activated (phosphorylated) NCC, hence leading to a high influx of sodium into the bloodstream [51, 52]. However, other studies have shown inverse correlations between sodium intake and total NCC and phosphorylated NCC expression [52].

1.11.2 Potassium

Potassium is the most abundant intracellular ion within the human body [53], with a higher intracellular concentration than the concentration in the extracellular matrix. The electrolyte is in such high abundance that the potassium is thought to have a greater regulatory effect on blood pressure than sodium and volume, and may explain why potassium has many different regulatory roles of blood pressure [53, 54]. For example, the stimulation of Na^+-K^+ ATPase causes vasodilation within the smooth muscle of arteries [53].

Normal physiological serum potassium concentration ranges between 3.5-5.0mmol/L, with lower concentrations termed hypokalemia and higher termed hyperkalaemia. It is assumed that at steady state, daily intake of dietary potassium is excreted within 24 hours via the kidneys [55]. Therefore, a 24hr urine sample provides an excellent resource for researching the dietary intake and renal excretion of dietary potassium.

There is overwhelming evidence suggesting that increasing dietary potassium causes a decrease in blood pressure, ranging from rodent to human studies [53-58]. Some examples include rat models with spontaneous hypertension or a sodium-loaded diet, which all show a decrease in blood pressure with increased dietary potassium [56, 57], whilst human studies have shown with potassium supplementation, systolic and diastolic pressure reduce over a short period of time [58, 59].

One study in Georgia showed African-Americans had higher blood pressure than Caucasians, despite the similar NaCl consumption [53]. The consumption of potassium was significantly lower in the Africa-American population, suggesting that reduced potassium intake may lead to increased blood pressure regardless of the sodium intake [53].

The Dietary Approach to Stop Hypertension (DASH) diet study involved hypertensive volunteers increasing fruit and vegetable intake (a natural source of high potassium concentrations) [60]. The results showed a decrease in systolic pressure, suggesting the increased potassium concentration affects the regulation of blood pressure [60].

However, it remains unclear whether it is solely potassium that is responsible for this change in hypertension, or a combination of other constituents of the DASH diet such as increased dietary fibre and low sodium context [60, 61].

1.11.3 Chloride

In addition to sodium, chloride is thought to have an inhibitory effect on WNK1 and WNK4, by binding to a specific chloride binding site. With increased intracellular chloride concentrations, this leads to a reduction in autophosphorylation (activation) of WNK1 and WNK4 [32].

Rats injected with sodium bicarbonate had a limited increase in blood pressure when compared to those injected with sodium chloride, suggesting it is plasma chloride also causes changes to blood pressure regulation [62].

1.11.4 Calcium

Recently, publications have claimed a synergistic link between Ca^{2+} and K^{+} through the calcium and calmodulin induced calcineurin protein. Using mouse models, Shoda *et*, al. predicts that calcineurin dephosphorylates NCC, therefore deactivating the cotransporter [63]. Studies have also shown the reverse of Shoda *et*, al.'s study, by blocking calcineurin with tacrolimus [64]. This inhibition causes NCC to activate and increase blood pressure [64]. It seems that calcium and calcium-dependent proteins play a much larger role in regulating blood pressure than previously thought. Calcineurin inhibitor-induced hypertension, therefore, shows similarities with Gordon syndrome.

1.12 Thiazide-Induced Hyponatremia

1.12.1 Current treatment of Hypertension

The treatment of hypertension usually involves lifestyle modifications and anti-hypertensive medications. Within the UK, national guidelines formulated by The National Institute for Health and Care Excellence (NICE) and the British Hypertension Society have been based on analysis of the efficacy and cost of antihypertensive drug categories [4]. Individuals, depending on their situation e.g. age and ethnicity are started on one drug e.g. an ACE inhibitor.

The NICE guidelines aim to reduce blood pressure to below 140mmHg/90mmHg (or 150/90mmHg if aged over 80 years) through a particular algorithm, illustrated in Figure 1-5. The starting treatment depends on a person's age and ethnicity, but if blood pressure does not reach the target, combinational therapy is introduced regardless of how old a person is [65].

1.12.2 ACE inhibitors

Angiotensin-Converting-Enzyme (ACE) inhibitors are the first treatment choice for patients below the age of 55 [65]. The drug is taken orally and acts by inhibiting the RAS and increasing the production of prostaglandin. It's a complex mode of action causes vasodilation, hence reducing the constriction of blood vessels and decreasing blood pressure [66]. The most common side effect of taking ACE-inhibitors is a cough. However other side effects are dose-dependent, including skin rashes, fever and neutropenia [66]. Where ACE inhibitors are not tolerated, Angiotensin II receptor blockers are used [67].

1.12.3 Calcium-channel blockers

Calcium-channel blockers are used for patients that are over the age of 55 [65]. Calcium-channel blockers bind to the L-type calcium channel found in smooth muscle tissue [68]. This inhibition causes vasodilation of circulatory vessels, leading to lower blood pressure [68]. This drug type is extremely successful at reducing cardiovascular diseases i.e. stroke and heart disease, however, there is a small increase in heart failure [68]. If calcium-channel

blockers do not reduce blood pressure below the target, ACE inhibitors are used in addition to calcium-channel blockers [65].

1.12.4 Thiazide-like diuretics

Thiazide diuretics have been used clinically since 1958 [69]. Their mechanism of action is by inhibiting the thiazide-sensitive NCC. This causes urinary dilution and natriuresis (renal salt wasting), causing arginine vasopressin (AVP) secretion [70]. The resulting reduced blood pressure is a combination of all these sequential events, as well as water retention within the CD and the loss of sodium and potassium [32, 70-72]. Thiazides can be administered as a monotherapy, however, most patients with essential hypertension require other anti-hypertensive drugs, e.g. ACE inhibitors and dihydropyridine calcium-channel antagonists, to control blood pressure to target [4, 6, 71]. Although thiazides are an effective treatment for control blood pressure, they can cause Thiazide-Induced Hyponatraemia (see below) [70].

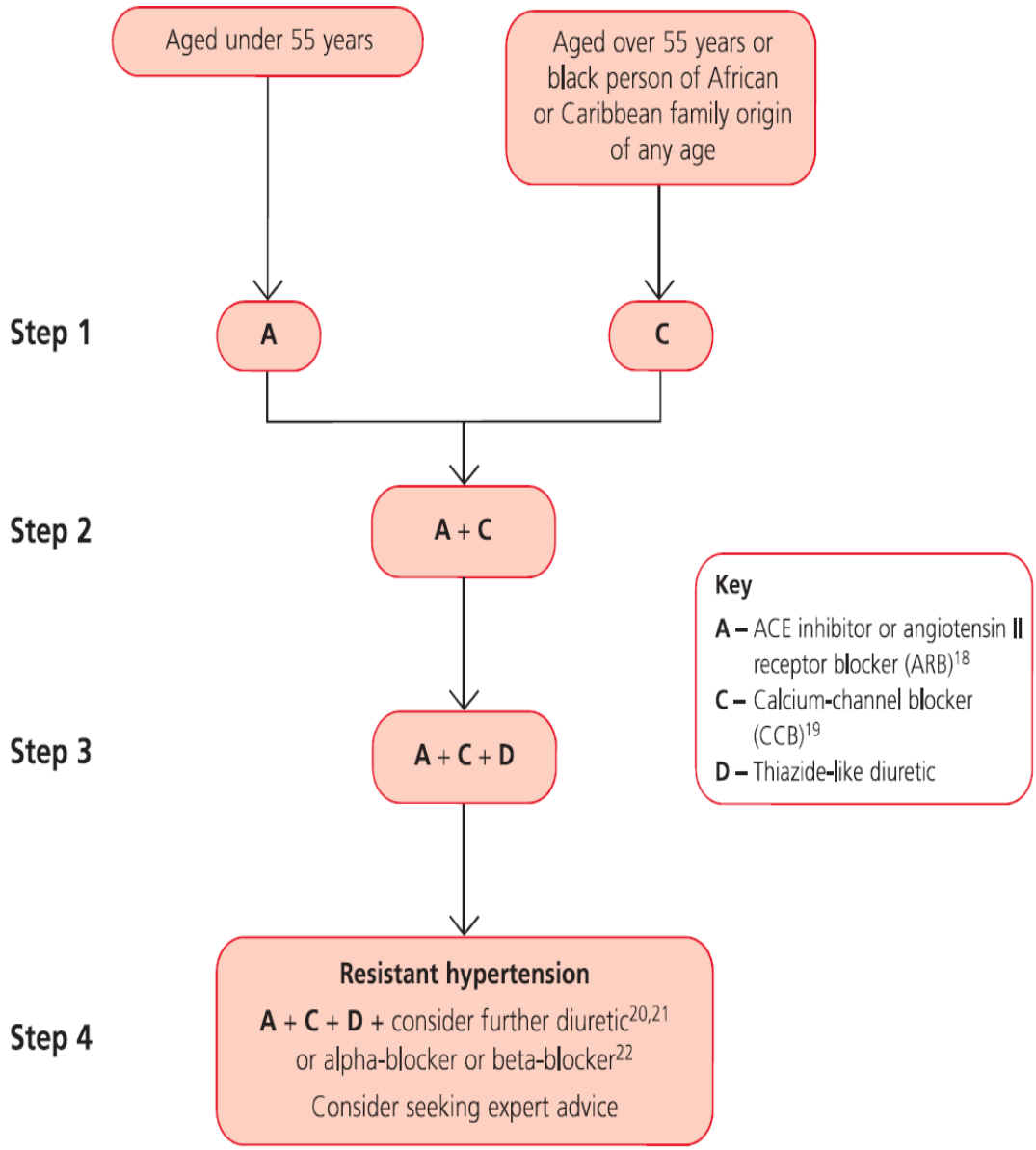


Figure 1-5 NICE Guideline CG127 on hypertension drug treatment.
Depending on their age and ethnicity, patients begin anti-hypertensive treatment either ACE inhibitors or Calcium-channel blockers. Failure to control blood pressure leads to escalating combination therapy of ACE inhibitors and Calcium-channel blockers. Thiazides are only offered at step 3 (or step 2, if either of A or C cannot be tolerated). If blood pressure does not reach before 140mmHG/90mmHg, resistant hypertension is diagnosed. Only changing different types of the same drug can be offered. Figure reproduced from NICE guideline CG127 [65].

1.12.5 The Causes of Hyponatremia

Hyponatraemia must result from the dysregulation of sodium and/or water transport within the distal nephron, causing either natriuresis or excessive water reabsorption and so diminished plasma sodium concentration [72]. The most common causes of hyponatraemia are drug-induced, Syndrome of Inappropriate Antidiuretic Hormone (SIADH), or heart failure [73]. Depending on both the absolute concentration of sodium and the rate of fall of sodium concentration over time, the severity of symptoms may vary. If sodium is >130mM, then hyponatraemia is usually asymptomatic [74]. However, the lower the sodium concentration and the more rapid the fall in sodium concentration, the more likely acute symptoms are to develop [75]. These include nausea, vomiting, confusion, falls, cerebral oedema, and seizure [72, 75, 76].

1.12.5.1 Thiazide-Induced Hyponatraemia

The most common cause of drug-induced hyponatraemia is Thiazides [75]. Although thiazides are effective and well tolerated by most, a minority of patients (14%) develop Thiazide-Induced Hyponatraemia (TIH) [75]. This is defined as a serum sodium concentration <135mM caused by a thiazide diuretic. TIH usually develops within days; it can occur within hours or sometimes occurs many months or even years into therapy [74, 77]. The reason for such variability in the duration of treatment and time to TIH is not understood.

TIH can even be sometimes fatal, but TIH has not been shown to increase overall mortality within the population of patients prescribed thiazides. The primary cause of fatality in TIH is cerebral oedema which leads to brainstem herniation causing mechanical compression of vital midbrain structures, although fatal trauma from falls and confusion can sometimes occur [78].

There are several hypotheses for the mechanism of TIH. Some reports suggest that TIH could develop due to an increase in the total amount of water carried within the body [71]. Others predict that TIH is caused by inappropriate secretion of AntiDiuretic Hormone (ADH) or increased sensitivity towards the hormone. The most consistently reported

demographic features of patients with TIH have increased age and female gender hence the typical patients with TIH are women over 75 [74, 79]. It is unclear, however, whether either association is causal since the elderly are those who are most likely to be prescribed thiazides, and older cohorts are predominated by females due to their longer life expectancy than males.

Recently, a study found a possible mechanism causing TIH in some patients [77]. The Prostaglandin transporter (PGT), known for its role in renal water reabsorption, was shown to have a variant (*SLCO2A1* p.A396T), which *in vitro* reduced PGT activity in its phosphor-mimic form [77]. This reduced activity may be responsible for the symptoms seen in patients with TIH who have the PGT variant [77]. Although only half of TIH patients expressed the PGT variant, it suggests an association of PGT with TIH in at least some patients. It was also concluded that there were likely to be other genetic and environmental components causing the development of TIH in patients without the PGT mutation [77].

Treatment of TIH depends on the severity. If symptoms are mild and not life-threatening, alternative anti-hypertensive medication is substituted for the thiazide diuretics [75]. TIH is expected to correct itself in 1-5days [75]. Acute TIH (severe: sodium <125mmol/L) patients require hospital care, but only those presenting symptoms of seizures or altered consciousness require a Hypertonic (3%) saline solution. Intravenous sodium is administered, increasing concentrations slowly over the first hours (1-2mmol/L/h), but reducing the rate of correction to 12mmol/L over 24hours [75]. Overly rapid correction of hyponatraemia can lead to central Pontine Myelinolysis, an often-fatal condition.

1.12.5.2 Syndrome of Inappropriate Antidiuretic Hormone

SIADH develops secondary to an underlying condition e.g. small cell carcinoma (lung malignancy) or drug administration [80]. The clinical presentations and serum and urinary biochemistry of SIADH are similar to TIH, therefore diagnosis can often be difficult [81]. However, Bartter & Schwartz identified key criteria for the diagnosis of SIADH [82]:

- Decreased plasma osmolality
- Severely concentrated urine
- Euvolemic
- Increased urinary sodium
- No diuretic use

Although clinical presentations are similar between TIH and SIADH, treatment is very different. Firstly, TIH patients stop the medication of thiazide diuretics and rehydrate with intravenous saline, which usually reveals which condition the patient is suffering from. However, with patients suffering from SIADH, the usual course of treatment is to restrict fluids and administer hypertonic saline [83].

1.12.5.3 Heart failure

The development of hyponatraemia, caused by heart failure, usually presents in the later stages of the disease [84]. Heart failure patients exhibit lack of renal water excretion, with increased secretion of ADH, as well as other hormones e.g. angiotensin II and renin [85]. The decreased cardiac output in heart failure patients causes the body to retain water and sodium so that blood pressure is preserved [84].

Heart failure hyponatraemia can arise by salt-restricted diets or the use of loop diuretics [84]. These increase the sodium and potassium stores within the body, predisposing patients to the development of hyponatraemia [84].

The usual management of hyponatraemia in heart failure patients involves fluid restriction and sometimes V2R antagonists (e.g. Tolvaptan), but there are no specific guidelines [85]. Where the administration of saline is used in TIH and SIADH treatment, heart failure patients cannot receive this treatment due to the associations with volume expansion [85].

1.13 Using Extracellular Vesicles to study renal pathology

Extracellular vesicles (EV) are phospholipid structures containing macromolecular structures from the cell of origin [86]. Currently, EVs can be categorized into 3 sub-species: Microvesicles (50-250nm), Apoptotic bodies (50-5000nm) and Exosomes (20-50nm) [87]. Extracellular vesicles can be differentiated from each other by their size and from the origin of the cell (Figure 1-6). Due to the overlap of size between the EVs, isolating specific populations of the vesicles is difficult. However, specific biomarkers can help to identify the EV of interest.

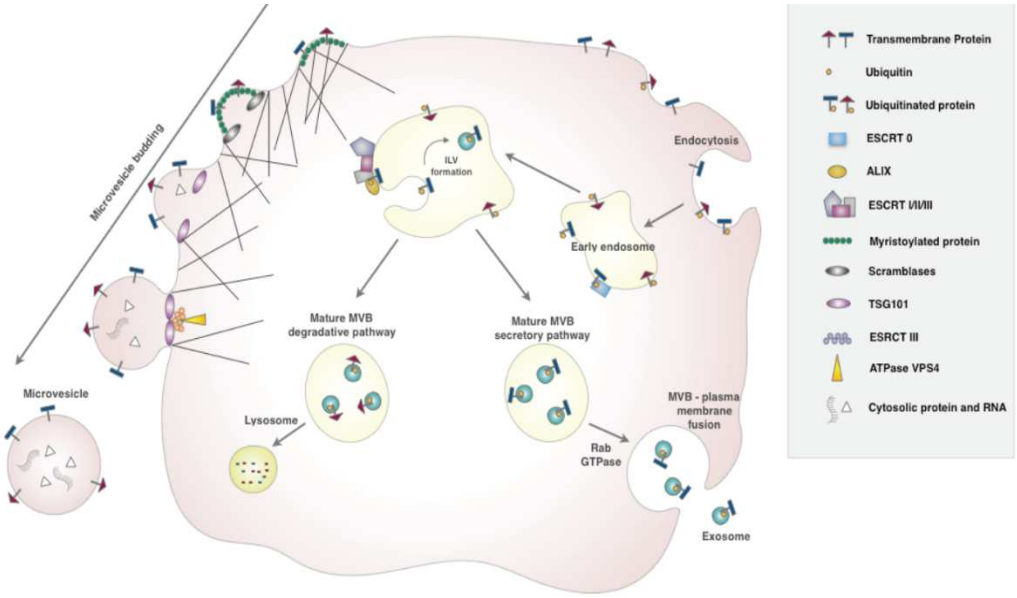


Figure 1-6 Production of exosomes and microvesicles. *The production of microvesicles (left) differs from the production of exosomes (right). Whilst microvesicles are formed through the budding of the phospholipid cell membrane with transmembrane proteins intact, exosomes are produced through a complex internalisation process. Transmembrane proteins are endocytosed, creating an early endosome. The cytosolic domains of proteins are bound by ubiquitin for cleavage and degradation. ESCRT proteins and ALIX recognise internalised ubiquitinised proteins to and begin the formation of Intraluminal vesicles (ILV). At this point, transmembrane proteins and other macromolecules (other proteins, DNA RNA) are internalized inside the newly formed ILVs. With the ILVs, the mature multivesicular bodies follow either the degradative pathway, where exosomes are degraded by lysosomes or the secretory pathway. The multivesicular bodies fuse to the cell membrane releasing the exosomes into the extracellular fluid. It is thought that transmembrane proteins, as well as other exosomal content, determine whether multivesicular bodies follow the secretory or degradative pathway [88-90].*

1.13.1 Microvesicles

Microvesicles, also known as ectosomes, are created through "budding" of the cell membrane, giving them a heterogeneous characteristic [88, 91]. There is an overlap in size between exosomes and microvesicles, therefore their formation and content are used to distinguish between the two EVs e.g. transferrin is absent in microvesicles, whilst highly abundant in exosomes [92].

ADP-ribosylation factor 6 (ARF6) stimulates a signalling cascade to cause the rearrangement of the cytoskeleton and the phospholipid membrane [92]. The microvesicles are released upon the phosphorylation of myosin light chains [92]. This formation allows the internalisation of a small amount of cytoplasm, but the majority of the molecules found within the microvesicles are that of the cells membrane [92].

1.13.2 Apoptotic bodies

Apoptotic bodies (or blebs) are the largest vesicles produced from a cell. Unlike microvesicles and exosomes, apoptotic bodies are only formed during fragmentation, the latter stages of apoptosis [93]

Despite containing cytoplasm and organelles, contents of these EVs are not consistent, as they include degraded material of different sections of the apoptotic cell [93]. Identification of these EVs involves fragmented DNA and histones [93].

1.13.3 Exosomes

Exosomes are the smallest of the EV described and are excreted by exocytosis at the apical membrane from the majority of cell types e.g. red blood cells, renal epithelia, saliva mucous cells, breast alveoli and pleural epithelia [94-96]. As with the other EVs, they contain specific macromolecules from their cell of origin, making them an excellent resource of research. However, exosomes are considered homogeneous in the "cargo" that they contain [88]. Exosome analysis is a non-invasive method to allow the study of the pathophysiology of many types of organs. This provides a huge clinical potential in facilitating rapid diagnosis e.g. Adult Polycystic Kidney Disease

(ADPKD) where early treatment may prevent progression of renal failure; early diagnosis may be possible due to biomarkers present only within exosomes [97].

Exosome vesicles have the potential to provide a vast amount of information because they contain proteins, lipids, nucleic acid, transcriptional factors, and carbohydrates from the cell. Whilst the majority of studies have analysed exosomal proteins and miRNAs, mRNA analysis may also be informative and provide additional, complementary data [98].

It has been well established that vesicles are used to transport molecules between intracellular organelles. Whilst the functions of exosomes are currently unknown, it is hypothesised that they are also involved in communication between cells [99]. Other functions of exosomes also include nonclassical protein secretion pathways, immune system functions, pathogen biology and roles in cancer progression [97, 100]. Functional studies using cell biology would clarify whether exosomes have a specific function, or if they are merely waste vesicles containing material from the cell of origin [101].

1.13.4 Urinary Exosomes

Urine is composed of water, proteins, glucose, amino acids, urea, and electrolytes including Na^+, K^+, HCO_3^-, Ca^{2+} and Cl^-. Urinary proteins can be separated into two categories: soluble proteins and solid-phase elements [94]. The solid-phase elements can be separated by ultrafiltration into sediment precipitated proteins and exosomes [94]. Urinary exosomes have attracted much attention since their discovery in 2004 by the Knepper group [98].

Currently, the most efficient way of isolating exosomes from urine is through ultracentrifugation. However, this is a time consuming and expensive method. A number of groups have found an alternative in using ultrafiltration, which gains a substantial yield of high-quality protein adequate for use in experiments [102-104].

Urinary Exosome analysis may be a novel method for studying FHHt and TIH, enabling an abundant and non-invasively acquired biological material to study the renal expression of NCC and other cotransporters [49].

1.13.5 Exosomes and Biomarkers

Exosomes are emerging as a useful material to identify real-time molecular mechanisms of their cells of origin [88]. These molecular mechanisms can be measured by using biomarkers, some of which cannot be found in traditional tissue samples. Biomarkers within exosomes can be used to diagnose diseases at an early stage, allowing for faster intervention e.g. Prostate-Specific Antigen (PSA) found in urine to allow the diagnosis of prostate cancer [105].

1.13.5.1 Tamm-Horsfall protein

The Tamm-Horsfall Protein (THP), also known as uromodulin, is the most abundant urinary protein (30-60mg/24hrs in humans is excreted) [7, 106, 107]. Although mutations in THP can cause familial juvenile hyperuricemia and medullary cystic kidney disease type 2, its physiological role in healthy people remains unclear. However, THP is of practical importance in urinary exosome experimental methodology as it can cause significant interference when extracting exosomes and prevent reproducibility in Western blots [108].

1.13.5.2 Aquaporin-2

Amongst the aquaporin channels that are permeable to urea and ammonia, Aquaporin (AQP0-4) channels are also water permeable. They are one of the major routes by which water is reabsorbed from the apical and basolateral membrane [94, 109]. AQP2 is highly abundant along the nephron and in urinary exosomes, and therefore may be an important marker for kidney diseases involving dysregulation of DCT water trafficking [94, 110]. The protein has also been linked to upregulation in TIH patients, due to the reduced free water clearance and the increased water retention [75].

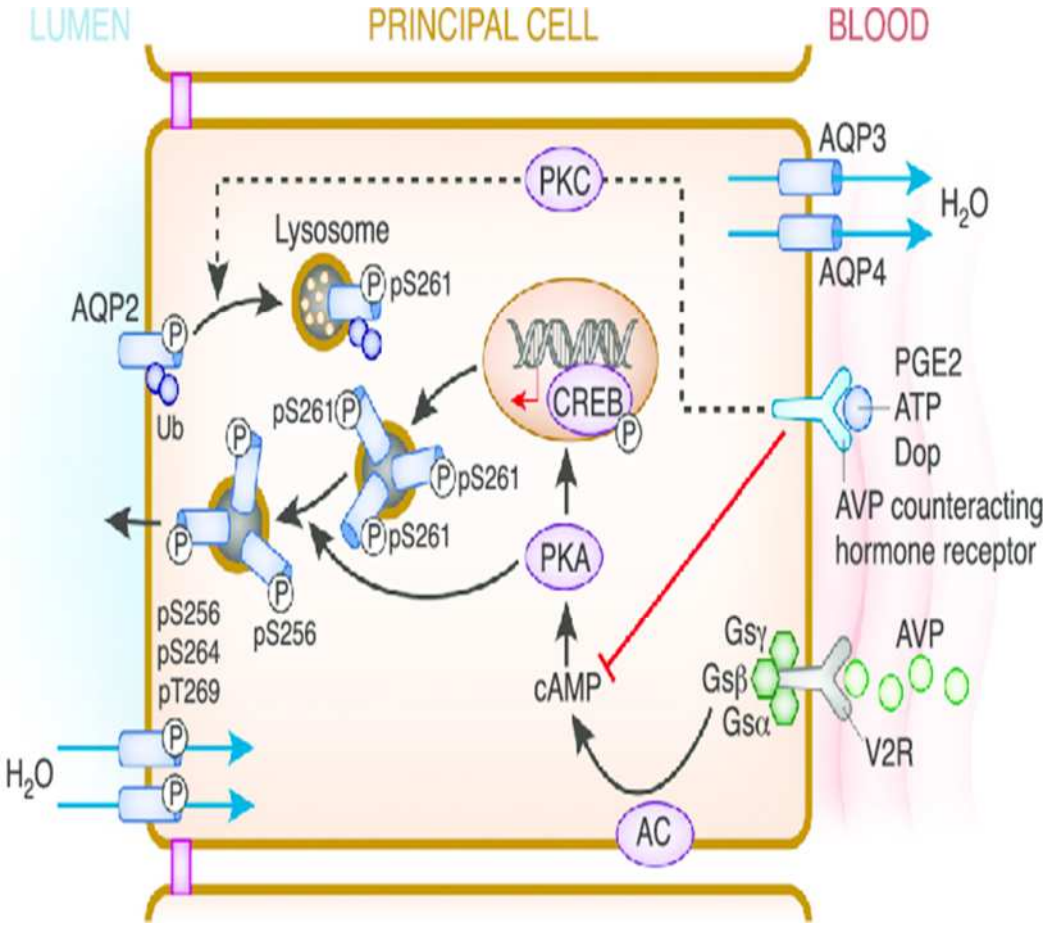


Figure 1-7 The regulation of AQP2 by the cAMP cascade. *cAMP is phosphorylated by the Gs complex after Vasopressin (AVP) has bound to its receptor (V2R). Downstream, AQP2 is transcribed and translated, before transferring to the apical membrane. Through the process of ubiquitination and lysosomes, AQP2 is degraded from the cell membrane surface. Once ubiquitinised, AQP2 is phosphorylated again and transported back to the cell membrane. AQP2 is active when phosphorylated, allowing the entry of water molecules [15, 111].*

AQP2 is principally regulated by the hormone vasopressin (also known as ADH) using a mechanism involving a cAMP cascade leading to PKA phosphorylation of AQP2 [109]. This close relationship creates a tight correlation between the expression of AQP2 on the exosomes and vasopressin levels by which water retention is caused by elevated circulatory levels of vasopressin [1, 112].

1.13.5.3 ALIX

ALG-2-interacting protein X (ALIX) is a high conserved adaptor protein found within the cytoplasm. Its role within the cell is believed to be the regulation of several apoptotic signalling pathways [113, 114]. Within the cell, ALIX specifically binds to a calcium protein called ALG-2 (Apoptosis-Linked Gene 2), with its inhibition preventing cell death [114]. In addition to its cellular function, recent studies have shown that ALIX could be linked to the packaging and formation of exosomes [115].

Recent publications have used ALIX as an exosomal marker, due to its non-functional role within the areas of interest and its heavy concentration within urinary exosomes because of its role in producing exosomes [116-118].

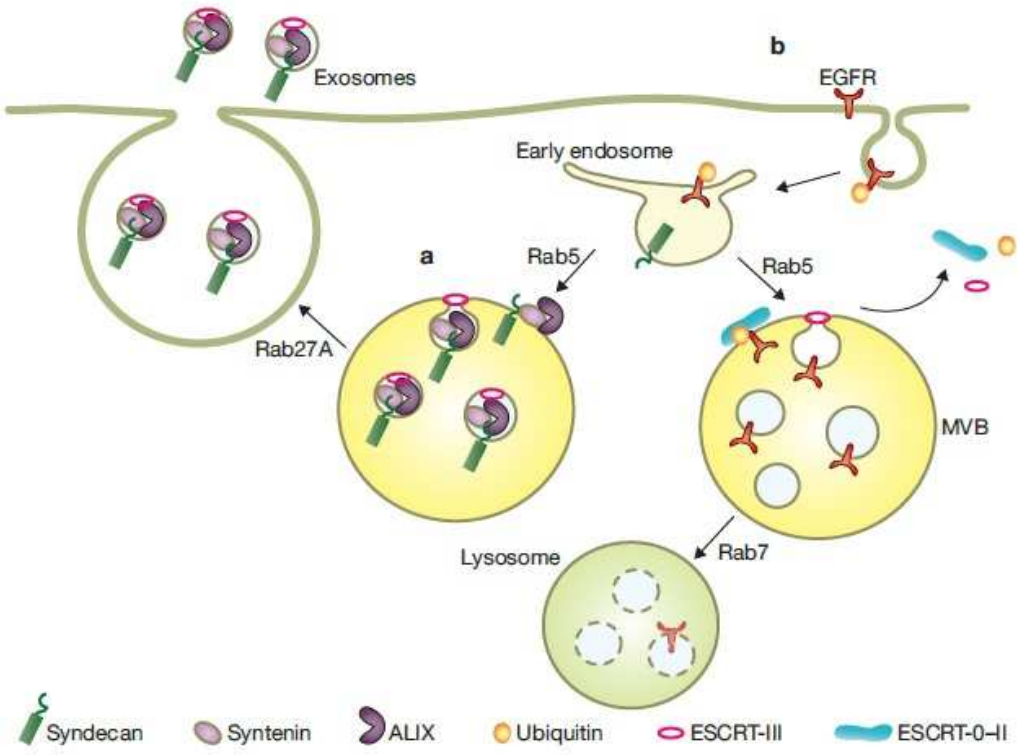


Figure 1-8 Function of ALIX in production of exosomes. *ALIX recruits the ESCRT complex to form ILVs, by binding to the external protein, Syntenin. ALIX remains bound to Syntenin throughout the multivesicular body secretory pathway. The membrane of the multivesicular body fuses to the cell membrane, mediated by the Rab27A complex pathway. The exosomes are released with ALIX still bound externally [115].*

1.13.6 Exosomal intercellular communication

Extra-cellular vesicles are comprised of exosomes and are believed to have many functions [119]. However, this is yet to be confirmed, but one possible role is to communicate between the exosome-producing cells and target cells. The purpose of this communication is also unknown but lipids and proteins are thought to transfer, and exosomes provide a stable environment to allow this type of communication [87, 120]. Exosomes can be distinguished by their RNA and protein profiles, which in turn may affect the gene expression of target cells. Some of these genes affected by certain exosomes are enzyme regulator activity, DNA replication, and ribosomal proteins [119]. Exosomes may interact with their target cells through receptor ligands; therefore, a specific cell stimulation is needed for the transfer of the exosomes' "cargo". However, if the cell stimulation is present and exosomes are internalised, lysosomes can still engulf exosomes that have not combined with endosomes. This also raises further questions as to whether there are further internal stimulations required for the exosomes and endosomes to exchange the "cargo".

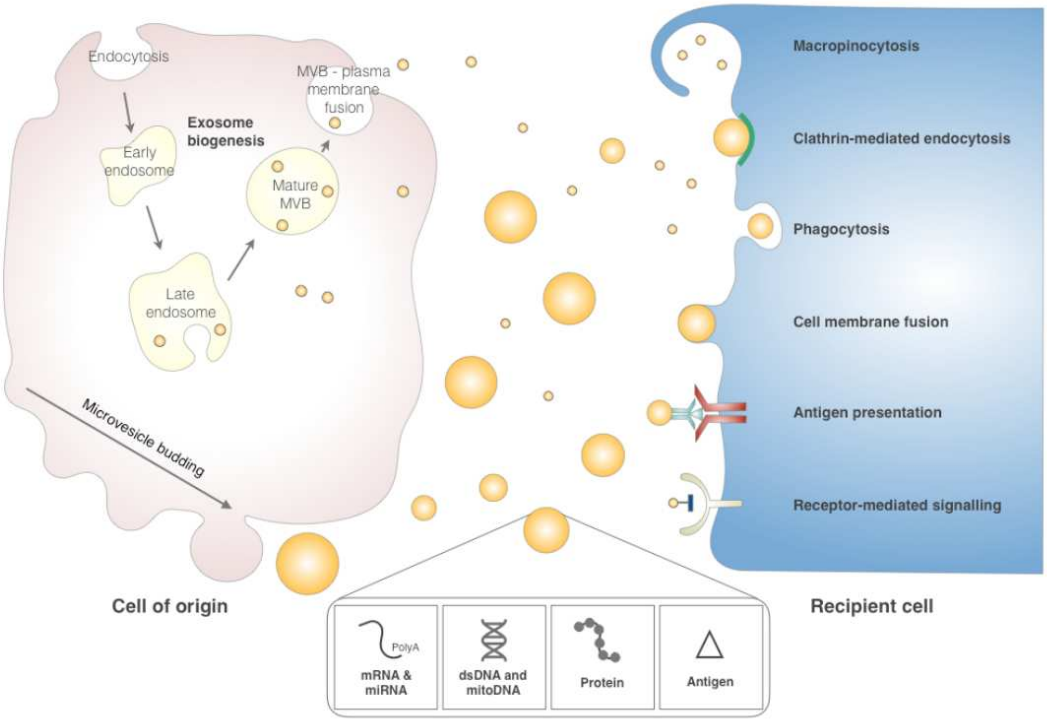


Figure 1-9 Microvesicles and exosomal communication with a recipient cell. *After the formation of EVs (see Figure 1-6), they are released into the extracellular fluid, where they are able to perform various communications with another cell (recipient cell). EVs can communicate through different interactions, including antigen presentation/receptor-mediated signalling (a complimentary attachment between an antigen and a receptor), or macropinocytosis (a form of non-specific endocytosis). EVs can also be internalized (Clathrin-mediated endocytosis) and/or degraded (phagocytosis). All interactions have the potential to propagate signals within the cell and changing their function [90].*

1.13.7 Exosomes use for understanding kidney disease

In addition to their basic biological role as explained above, exosomes are used to deepen the understanding of diseases, due to their reflection pathophysiological processes [121]. Some of these include diseases of the immune function, cardiovascular, kidney and neurodegenerative diseases. Studies have shown that these diseases change biological mechanisms like gene expression [121].

Recently, exosomes have been identified as a causative agent for the development of cancer, by cancerous cells releasing exosomes into their microenvironment inducing angiogenesis and a pre-metastatic niche [122, 123]. These exosomes can also spread into the bloodstream, causing metastasis. They have also helped explain how some types of cancers can spread across the blood-brain barrier [124].

Additionally, exosomes are able to diagnose Acute Kidney Injury (AKI) significantly earlier, which helps modifications to treatment [125]. It is thought that with cellular injury, it has been shown that there is an increase in the abundance of exosomes, as well as containing biomarkers for diagnosis e.g. Activating Transcription factor 3[125].

As well as diagnosing, exosomes have been used in clinical trials as an immunotherapeutic agent for treating cancer. The exosomes are designed to target cancer and have been shown to eradicate cancer completely in murine studies [126].

1.14 Aims, Hypothesis and Objectives

Cardiovascular disease is the most common cause of death worldwide and hypertension is one of its most important reversible risk factors. As thiazide-sensitive sodium trafficking is one of the principal determinants of human blood pressure it is vital to further understand and characterise the molecular mechanisms of this pathway.

To achieve this, two clinical paradigms (Gordon syndrome and Thiazide-Induced Hyponatraemia) of dysregulation of thiazide-sensitive renal sodium handling will be studied and compared to control patients (unaffected). The relationship between serum and 24hr urinary potassium and NCC expression in humans will also be addressed.

Characterisation of NCC will be achieved through the extraction of exosomes of urine and media from patients and cell lines respectively.

This project will address this area of research specifically by:

- Optimising extractions of:
    - Exosomes from the urine of patients
    - Exosomes from cell culture media
    - Protein from exosomes
    - mRNA from exosomes
- Conducting a physiological study of the relationship between serum K^+ and 24h dietary K^+ intake and the exosomal expression of NCC, pNCC, WNK4 and SPAK in the kidney
- Identifying target mRNA in urinary exosomes using the optimum extraction method
- Presenting differential expression of *SLC12A3* and related genes between healthy and TIH participants
- Studying communication of NCC-rich exosomes between HEK293 and primary epithelial Proximal Tubule Kidney cells.

Chapter 2: Materials and Methods

2 Methods and Materials

2.1 Clinical recruitment

The recruitment of patient and control samples were identified and detailed in each chapter. Spot urine samples were collected in 50mL universal containers from normonatraemic non-thiazide controls (healthy volunteers). 24hr urine samples were collected in a 3000mL collecting flask from acute, convalescent and normonatraemic patients. The following clinical features were recorded: blood pressure, heart rate, weight, height, age and ethnicity. Four 8mL vials of blood (Serum Separator Tube (SST), Plain, EDTA, and fluoride) and urine samples (spot and 24-hour) were collected from normonatremic non-thiazide controls. All volunteers and controls gave written consent, with the study following the standards of Good Clinical Practice sections 8.2.8, approved by the Local Research Ethics Committee (REC) (see Appendix 9.3 for Ethical Committee Approval).

2.2 Analysis of biochemical electrolytes

Samples were sent to the biochemistry department at the Nottingham University Hospitals NHS Trust to analyse for the various physiological factors listed in (Table 2-1). Samples were received at sample reception and spun down for 10-15 minutes before being placed on Chemical Bioanalyser: Sysmex and Ilympus (blood and urine, respectively). Unless abnormal results were produced, for example, hyperkalaemia or hyponatraemia results from the samples were sent straight to the laboratories system (WinPath), which is linked to Notis (the hospital's system for result collection). Abnormal results were checked by a Clinical Scientist before sending through to WinPath. Old samples were stored up to 5-10 days at -80°C before they were discarded.

Once the analysis was complete, 2x 20mL universal Falcon tubes were aliquoted from the remaining 24hr urine and sent to the research laboratory within 6 hours of delivery to the NHS Biochemistry department. The quality of the exosomal protein and the RNA is likely to be affected during this brief time, however Protease inhibitors (cOmplete, Mini, EDTA-free®- Roche) were

added to each falcon tube and stored in -80°C freezers upon arrival at the research laboratory until ready for further processing.

Table 2-1 Equipment used to measure electrolytes, vitamins and hormones of TIH patients. *Above describes the different methodology used to measure the serum and 24hr urine concentrations of the parameters. Only electrolytes were measured in 24hr urine. This table was taken from the supplementary section from Ware* et *al. [77].*

Biochemistry parameter	Method	Machine
Serum:		
Sodium	Indirect ion selective electrode	Beckman AU clinical chemistry analyser
Potassium	Indirect ion selective electrode	Beckman AU clinical chemistry analyser
Urea	GLDH kinetic UV test method	Beckman AU clinical chemistry analyser
Creatinine	IDMS-traceable kinetic colour test (Jaffe) method	Beckman AU clinical chemistry analyser
Osmolarity	Freezing point depression	3300 Micro-Osmometer
TSH	Sandwich immunoassay	Siemens Advia Centaur
Glucose	Hexokinase UV method	Beckman AU clinical chemistry analyser
Chloride	Indirect ion selective electrode	Beckman AU clinical chemistry analyser
corrected Calcium	Arsenazo III Photometric colorimetric test method	Beckman AU clinical chemistry analyser
Phosphate	Photometric UV test method	Beckman AU clinical chemistry analyser
Magnesium	Colorimetric test method	Beckman AU clinical chemistry analyser
Bicarbonate	Colorimetric enzymatic assay	Beckman AU clinical chemistry analyser
Zinc	ICP-MS	Agilent 7700x ICP-MS
Vitamin D	Immunoassay	Siemens Advia Centaur
Plasma:		
Renin	Chemiluminescent immunoassay	DiaSorin analyser
Aldosterone	Chemiluminescent immunoassay	DiaSorin analyser
PTH	Immunoassay	Siemens Advia Centaur
24h urine:		
Sodium	Indirect ion selective electrode	Beckman AU clinical chemistry analyser
Potassium	Indirect ion selective electrode	Beckman AU clinical chemistry analyser
Urea	GLDH kinetic UV test method	Beckman AU clinical chemistry analyser
Creatinine	IDMS-traceable kinetic colour test (Jaffe) method	Beckman AU clinical chemistry analyser
Chloride	Indirect ion selective electrode	Beckman AU clinical chemistry analyser
Calcium	Arsenazo III Photometric colorimetric test method	Beckman AU clinical chemistry analyser
Phosphate	Photometric UV test method	Beckman AU clinical chemistry analyser
Magnesium	Colorimetric test method	Beckman AU clinical chemistry analyser

2.3 Urinary Exosome extraction

2.3.1 Ultrafiltration

Samples, thawed overnight at 4°C, were centrifuged at 15,000g for 30 minutes at 4°C using an MS-Beckman Centrifuge. After centrifugation, the cell pellet from each sample was suspended in 2% SDS buffer and stored at -80°C. The supernatant was transferred to Vivaspin™ 20 centrifugal concentrator (Sartoris™) and centrifuged at 4,200g for another 30 minutes at 4°C. The samples were centrifuged until concentrated at 0.2µM and collected in 1.5mL Eppendorf tubes. The flowthrough samples from the Vivaspin™ 20 centrifugal concentrator were collected. All samples were stored in 2% SDS buffer in -20°C or -80°C.

2.3.2 Exoquick

Samples were thawed overnight at 4°C and vortexed thoroughly for 1 minute. Samples were centrifuged at 2500g for 5 minutes to remove cells. Once the supernatant had been removed, 1mL Exoquick-TC was added and gently inverted before vortexing. The samples were incubated overnight at 4°C. The following day, samples were centrifuged at 13000g for 30 minutes. The supernatant was discarded and 350µL lysis buffer was added to the exosome pellet. Samples were vortexed for 15 seconds and left at RTe for 5 minutes to allow complete lysis. Finally, 200 µL of 100% ethanol was added to the samples in the 1.5mL Eppendorf tube and vortexed for 10 seconds. All samples were stored at -80°C.

2.3.3 miRCURY

Urine samples were thawed overnight at 4°C and vortexed thoroughly for 3 minutes. Samples were centrifuged at 2500g for 5 minutes to remove cells before 4mL of precipitation buffer B was added. The tubes were gently inverted a few times, before vortexed briefly and incubated overnight at 4°C. After incubation, samples were centrifuged at 3200g for 40 minutes at 20°C. The supernatant was discarded and 350µL lysis buffer was added to the exosome pellet. The re-suspended exosome pellet was transferred to a sterile 1.5mL Eppendorf tube, vortexed for 15 seconds and left at RTe for 5 minutes.

Finally, 300µL of 100% ethanol was added into the Eppendorf and vortexed for 10 seconds. All samples were stored at -80°C.

2.3.4 exoEasy

Urine samples were thawed overnight at 4°C and vortexed thoroughly for 1 minute. The vortexed samples were centrifuged at 2500g for 5 minutes at 4°C before the supernatant was transferred to fresh tubes. Next, 1 volume buffer XBP was added to 1 volume of sample. The two volumes were mixed well and gently, and left aside at RTe, for 3 minutes. The mixture was added to the exoEasy spin column and centrifuged for 1 minute at 500g. The flowthrough was discarded. 3mL buffer XWP was added to the columns and centrifuged at 3000g for 3 minutes. The collection tube and flow through were discarded. 700µL Qiazol was added to the filters and centrifuged at 5000g for 5 minutes at RTe. The lysate was transferred to a 1.5mL Eppendorf tube, vortexed, and left for 5 minutes. Next, 300µL chloroform was added and shaken vigorously for 1 minute. The mixture was left at RTe for 5 minutes before centrifuged for 30 minutes at 12000g at 4°C. The upper aqueous phase was transferred to a new tube (avoiding the interface). Finally, 2 volumes of 100% ethanol were added to the exosome fraction and mixed well by pipetting. All samples were stored at -80°C.

2.4 Quantification of urinary exosomal proteins by Western blotting.

2.4.1 SDS-PAGE Electrophoresis

To identify proteins by their molecular weight, Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used for separation by electrophoretic mobility. SDS-PAGE gels were made using the reagents outlined in Table 2-2 and Table 2-3 and assembled using Bio-Rad Mini-protean II gel electrophoresis apparatus.

Table 2-2 Volumes of components for composition of Resolving Gels. *The volumes shown below were to make 4 gels at one time. Different percentage gels were used depending on the molecular weight of the protein e.g. 7.5% was used for NCC, 10% was used for ROMK and 12.5% was used for AQP2*

Acrylamide resolving gel-components	7.5% Gel Volume	10% Gel Volume	12.5% Gel Volume
Distilled water	24mL	20mL	15.5mL
30% Acrylamide	12.5mL	16.5mL	21mL
10% SDS	500µL	500µL	500µL
1.5M TRIS-HCl (pH8.6)	12.5mL	12.5mL	12.5mL
10% APS	500µL	500uL	500µL
TEMED	20uL	20µL	20uL

Iso-propo-butanol was layered on the top of the resolving gel after the gel was pipetted into a gel making cassettes and allowed to set. The stacking gel (5%) was prepared (Table 2-3) and added to the resolving gel, with a 10-well comb placed between the glass plates.

Table 2-3 Volumes of components for composition of Stacking Gels. *As above, these volumes were to make 4 gels at a time. There was no variation in acrylamide concentration in the stacking gel, depending on the protein's molecular weight.*

Acrylamide Stacking gel components	Volume
Distilled water	13.6mL
30% Acrylamide	3.32mL
10% SDS	200µL
1.0M TRIS-HCl (pH6.8)	2.52mL
10% APS	200µL
TEMED	20µL

Samples were prepared in accordance to the Bicinchoninic Acid Assay (BCA) protein estimation (see section 2.6.2) and 2x sample buffer. Protein samples were denatured at 95°C for 5 minutes before resolving at 100V (7.5%=NCC/10% =SPAK & ROMK/12%=AQP2 & SPAK) for 2 hours in 1X Tris-HCl-SDS running buffer. This continued until just after the blue band, created by the 2x loading buffer, runs off the gel.

2.4.2 Western blotting using the wet technique

The gels were removed from the glass plates and assembled in a transfer cassette with blotting paper and PVDF membrane (activated by 100% methanol). The membrane was placed above the gel, to allow the transfer of the proteins onto the membrane (in accordance with their electrophoretic characteristics). The cassettes were placed facing the cathode in 1X Tris-HCl transfer buffer at 4°C and run at 60V for 2 hours.

Table 2-4 List of primary and secondary antibodies used. *The type of blocking agent used depended on the primary antibody that was used to target the protein of interest. Antibody incubation also varied due to the specificity and sensitivity of the primary antibody.*

Blocking agent	Primary Antibody	Antibody Incubation	Secondary Antibody
5% Marvel™ Milk	NCC_3 (1:500)	Overnight 4°C	Goat Anti-Rabbit HRP (1:10000)
5% Marvel™ Milk	$NCC_{1/2}$ (1:500)	Overnight 4°C	Goat Anti-Rabbit HRP (1:10000)
5% BSA	pNCC S91 (1:500)	Overnight 4°C	Donkey Anti-Human HRP (1:10000)
5% BSA	pNCC T55 (1:500)	Overnight 4°C	Donkey Anti-Human HRP (1:10000)
5% BSA	pNCC T60 (1:500)	Overnight 4°C	Donkey Anti-Human HRP (1:10000)
5% Marvel™ Milk	ROMK (1:500)	Overnight 4°C	Goat Anti-Rabbit HRP (1:10000)
5% Marvel™ Milk	SPAK (1:500)	Overnight 4°C	Goat Anti-Rabbit HRP (1:10000)
5% Marvel™ Milk	WNK1 (1:500)	Overnight 4°C	Goat Anti-Human HRP (1:10000)
5% Marvel™ Milk	WNK4 (1:500)	Overnight 4°C	Goat Anti-Rabbit HRP (1:10000)
3% Marvel™ Milk	M2, FLAG (1:1000)	30 minutes	Anti-Mouse HRP (1:10000)
5% Marvel™ Milk	AQP2 (1:500)	1hr room temperature.	Goat Anti-Rabbit HRP (1:10000)
5% Marvel™ Milk	ALIX (1:500)	1hr room temperature.	Goat Anti-Rabbit HRP (1:10000)
5% BSA	CD9 (1:500)	1hr room temperature.	Goat Anti-Rabbit HRP (1:10000)
5% BSA	B-actin (1:500)	1hr room temperature.	Goat Anti-Rabbit HRP (1:10000)

Once the transfer was complete, the membranes were blocked in non-specific protein solution (milk/BSA-TBST), before incubating the membranes in primary antibodies (Table 2-4). To remove any excess primary or secondary antibodies, the membranes were washed in TBS+0.1% v/v Tween (TBS-T) three times, each wash lasting 15 minutes.

After the primary antibody incubation and three lots of TBS-T washes, the membrane was incubated in HRP-conjugated secondary antibody.

2.4.3 Western Blot Protein Detection by ECL Method

To detect the various protein bands on the membrane, the Enhanced Chemiluminescence (ECL) method (QE Healthcare) was used. The ECL solution binds to the secondary antibody via the conjugate, allowing an oxidation reaction of diacylhydrazide luminol. The luminol becomes excited by photons and emits light. The membranes were incubated for 5 minutes in the ECL solution (1:1 of solution A & B). Membranes were exposed to photographic films (Hyperfilm, GE Healthcare) for various times depending on the abundance of the protein. Table 2-5 shows the various times allowed to expose the membranes to the film.

Table 2-5 Exposure times for membranes. *This shows the time allowed for the light exposure of the membrane to photographic film. Time varied depending on the abundance of the protein.*

Protein targets	Time of exposure
NCC_3	1½ hours
$NCC_{1/2}$	15 minutes
pNCC S91/T55/T60	40 minutes
AQP2	5-10 minutes
ALIX	5-10 minutes
ROMK	13-20 minutes
SPAK	15-30 minutes
WNK1	30 minutes
WNK4	30 minutes
B-actin	5 minutes
CD9	5 minutes

After the exposure time, the photographic films were developed in Kodak® developer (1:5) and rinsed in Kodak® fixer (1:5) until transparent. This allowed the membranes to be visualised on the photographic film. The films were then rinsed in water, before air drying.

Using Image J (version 2), densitometries of protein bands were calculated and normalised against ALIX, AQP2 or β-actin.

2.5 RNA extractions

All RNA work was performed in an RNase free environment using RNase ZAP™.

2.5.1 miRCURY

MiRCURY columns were assembled, before samples were added to the columns and centrifuged at 14000g for 1 minute. The flow-through was discarded and the same collecting tube was retained. The process was repeated for the remaining lysate. To the filters, 400µL wash solution was added and centrifuged at 14000g for 1 minute. The flow-through was discarded and repeated. After the second wash, the collecting tube was discarded, and the filters were transferred to a fresh 1.5mL sterile Eppendorf. To the filters, 30µL elution buffer was added in the middle of the column, centrifuged at 200g for 2 minutes and then 14000g for 2 minutes. Exosomal RNA was recovered in the Eppendorf. Samples were stored at -80°C.

2.5.2 ExoRNeasy

To the RNeasy Minelute spin column, 700µL of the sample was added, then centrifuged at 8000g for 15 seconds at RTe. This was followed by adding 700µL of buffer RWT and centrifuging at 8000g for 15 seconds at RTe. After, 500µL of buffer RPE was added and centrifuged at 8000g for 15 seconds at RTe. 500µL of buffer RPE was added and centrifuged at 8000g for 2 minutes at RTe. The sample was centrifuged for 3 minutes at 13000g. 30µL of RNase free water was added and centrifuged at 200g for 2 minutes and 16000g for 2 minutes. All samples were stored at -80°C.

2.5.3 SeraMir

The SeraMir columns were assembled in collecting tube and the lysate (600µL) was transferred into SeraMir columns. The tubes were centrifuged 13000g for 1 minute. The flow-through was discarded and the same collecting tube was retained. The previous step was repeated for the remaining lysate. 400µL of wash buffer was added and centrifuged at 13000g for 1 minute. The flow-through was discarded. The wash step was repeated twice before the samples were centrifuged at 13000rpm for 1 minute. The collecting tube was discarded and a fresh 1.5mL sterile Eppendorf was assembled. 30µL elution buffer was added in the middle of the column. The samples were centrifuged at 2000g for 2 minutes and then again at 13000g for 2 minutes. RNA was recovered in the Eppendorf. Samples were stored at -80°C.

2.5.4 GenElute

The GenElute columns (red ring) were assembled and 700µL lysate was added to the filters. The tubes were centrifuged at 14000g at 15 seconds and the flow through was discarded. 500µL of wash solution 1 was added and centrifuged at 14000g at 15 seconds, followed by discarding the flow through. 500µL wash solution 2 was added and centrifuged at 14000g at 15 seconds, followed by discarding the flow through. 500µL wash solution was added and centrifuged at 16000g at 1 minute, followed by discarding flow through. The columns were transferred into a sterile 1.5mL Eppendorf. Finally, 30µL elution solution was added and centrifuged at 200g for 1 minute and then again at 16000g for 2 minutes. Samples were stored RNA at -80°C.

2.5.5 ZRNA isolation

Zymo-spin IC column and collection tubes were assembled and 700µL of each sample was transferred into the column. The tubes were centrifuged for 30 seconds at 13000g. The flow-through was discarded. Next, 400µL RNA prep buffer was added into the column and centrifuged for 30 seconds at 13000g. The flow-through was discarded before 700µL RNA wash buffer was added into the column. The tube was centrifuged for 30 seconds at 13000g. The flow-through was discarded. 400µL RNA wash buffer was added into the column and centrifuged for 1 minute at 13000g. The flow-through was

discarded and the column was transferred into a sterile 1.5mL Eppendorf. Finally, 30µL DNAse/RNase free water was added into the column and centrifuged for 1 minute at 2000g and again for 2 minutes at 16000g. Samples were stored RNA at -80°C.

2.6 RNA and Protein validation

2.6.1 NanoDrop®

The resulting RNA was detected using a NanoDrop® Technologies NanoDrop® ND-1000 Spectrophotometer. The Nanodrop program was loaded and the machine equalised. A blank sample (HPLC water) was added to the loading space (1µL) and measured. Once the machine was set-up, samples were vortexed and centrifuged. Each sample was measured (1µL) and recorded. Results were saved as PDF's and Excel-sheets.

2.6.2 Bicinchoninic Acid Assay (BCA)

To assay protein concentration using Bicinchoninic Acid Assay (BCA) estimation kit (Pierce, ThermoFisher), known protein concentrations were made fresh: 0 (blank), 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.6, and 2.0mg/mL, and pipetted into a clear flat-bottom 96-well plate. BCA solution A (contains sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide) and BCA solution B (contains 4% cupric sulfate) were combined to 50:1 and mixed gently.

Samples to be measured were diluted (e.g. 1;5 or 1;10) and plated into the clear flat-bottom 96-well plate. 200uL of A/B solution was pipetted into each well and incubated for 30 minutes avoiding any light.

The plate was then read using a Flexstation setting the absorbance of 562nm. According to the absorbance and the known concentrations, the concentrations of samples were calculated using the equation generated by the standard curve.

2.6.3 Ribogreen

A 1x TE buffer was prepared with 20x TE buffer and sterile water (RNase free). Using the 1x TE buffer, RG 2000x with RG buffer was made. RNA stock was made with concentrated RNA and 1x TE buffer, which was used to make the RNA standards (Table 2-6).

Table 2-6 RNA Standards. *Details to make six RNA concentrations to produce a standard curve, plus a blank. Volumes have been doubled to allow two wells for each standard, to calculate an average reading.*

TE (µL)	RNA Stock (µL)	RNA (ng/mL)
0	200	200
100	100	100
150	50	50
175	25	25
195	5	5
199	1	1
200	0	blank

100µL RNA standards were added in specified wells of a 96-well plate, followed by 100µL of 1xTE buffer. When plating samples, 100µL of 1xTE buffer was added in every well followed by adding 2µL of each sample into each well. Samples and Standards were plated in duplicates. Finally, 100µL of RG 2000x was added to each well with a sample and standard. The plates were incubated for 5mins at RTe away from light. The sample fluorescence was measured at excitation (500nm) and emission (525nm) wavelengths respectively. The standard curve generated from RNA standards were used to calculate RNA sample concentration.

2.6.4 Bioanalyzer

Once mRNA concentration had been determined by the Ribogreen assay, the RNA Integrity (RIN) score was calculated using the Agilent 2100 Bioanalyser (Deep-seq, University of Nottingham). The RIN score determined which samples would be suitable for sequencing, with a RIN score higher than 8 deemed acceptable.

As the mRNA concentrations determined by the Ribogreen assay showed low concentration, it was decided to use the Agilent RNA 6000 Pico kit (Agilent), due to its increased sensitivity.

In brief, all reagents were equilibrated to RTe for 20-30 minutes, protecting the dye from light. The pre-made filtered gel and RNA 6000 Pico dye concentrate were vortexed for 10 seconds and centrifuged. 1µL of RNA 6000 Pico dye concentrate was added to a 65µL aliquot of filtered gel. The mixture was vortexed and inspected for proper mixing. Tubes were centrifuged for 10 minutes at RTe at 13000g. A new RNA chip was placed on the station and 9µL of the gel-dye mix was pipetted at the bottom of the well (marked G). The plunger of the syringe was pressed down until it was held by the clip, before waiting exactly 30 seconds and then releasing the plunger. 5 seconds was allowed for the plunger to raise by itself, before slowly pulling the plunger back to the 1mL position. 9µL of the gel-dye mix was pipetted in each of the wells (marker G and marker CS). 5µL of the RNA 600 Pico marker was added into the ladder well and each of the 11 samples wells. To minimise secondary structure, samples were heated to 70°C for 2 minutes. The ladder aliquot was thawed and 1µL of the diluted RNA 6000 Pico ladder was pipetted into the ladder well, before pipetting 1µL of each sample into each of the 11 sample wells. The chip was placed on the IKA vortex mixer for 60 seconds at 2400rpm. Finally, the chip was placed in Bioanalyzer within 5 minutes of vortexing and read. Once the read was complete, a smear analysis was performed to determine the mRNA percentage.

The assay output of each kit produces RNA concentrations, RIN scores, ribosomal ratios, and an electropherogram including the ladder and sample result for each well.

2.7 Polymerase Chain Reaction

2.7.1 Synthesis of cDNA

Using Invitrogen's Superscript™ First-Strand Synthesis System for qRT-PCR, 5ng of exosomal total RNA was converted to single-stranded complementary DNA (cDNA) following the protocol as described provided by ThermoFisher. Random Hexamers (Invitrogen) were used for cDNA synthesis. Random Hexamers also allow for multiple sites for primers to align, creating multiple overlaps of cDNA with various nucleic acid lengths. The cDNA generated was

treated with RNase to remove any remaining RNA template. Two negative controls were used: Reverse Transcriptase (RT) negative and No Template Control (NTC). Table 2-7 shows the RNA/primer mixture components for each RNA sample using 0.5mL DNAse/RNAse free tubes.

Table 2-7 Reagents used to make RNA/primer Master Mix per sample. *The reagents of the primer Mastermix were added into a separate Eppendorf tube and made dependent on the number of samples to be plated. The total volume of the primer Mastermix was aliquoted once RNA dilutions (5ng) had been made. All reagents were kept on ice.*

Master mix components	Volume per sample
10mM dNTP mix	1µL
Random hexamers (50ng/µL)	3.5µL
DEPC-treated water	Up to 10µL
RNA	1-6.5µL

The RNA/primer mixture was incubated at 65°C for 5 minutes on the heated lid DNA Engine Tetrad 2 Cycler followed by incubation in ice for 1 minute.

Table 2-8 Reagents used for 2x Master Mix per sample. *All reagents added to an Eppendorf depending on the number of samples in the synthesis.*

Master mix components	Volume per sample
10X RT buffer	2µL
25mM $MgCl_2$	4µL
0.1M DTT	2µL
RNAseOUT™ (40U/µL)	1µL

Once the 2x Master Mix was made with the above reagents (Table 2-8), 9µL was added to each RNA/primer sample followed by gentle mixing and centrifuging. The samples were incubated at RTe (25˚C) for 2 minutes before SuperScript™ II RT (1µL) was added to each sample (except RT control). The samples were incubated at RTe (25˚C) for 10 minutes, followed by further incubation at 42°C for 50 minutes, and terminated at 70°C for 15 minutes. Samples were chilled on ice. The samples were centrifuged before the addition of RNase H (1µL) to each sample and incubated for a further 20 minutes at 37°C. Samples were stored at -20°C until required for qPCR.

2.7.2 Quantitative PCR using Taqman

Quantitative PCR (qPCR) was used to measure levels of gene expression of samples. Using the Taqman® method, qPCR was used to try and validate RNA sequencing to confirm the presence of mRNA within the urinary exosomes. Total fetal kidney RNA (Agilent Technologies) was used as a positive control. Due to the lack of a housekeeping gene in the literature, concentrations of every sample were standardised to 5ng/µL. Using data from RNA sequencing and previous proteomics work, primers were selected as potential housekeeping genes (Table 2-9) for positive controls of qPCR assays.

Table 2-9 Pre-designed Primers used with TaqMan qPCR. *The primers targeted specific boundaries of each gene. These measured the expression of genes of interest in RNA samples sequenced by Oxford Genomics. No assays were multiplexed.*

Target gene	Assay ID	Exon boundary	Amplicon length
NCC	Hs01027568_m1	23-24	67
ALIX	Hs00994345_m1	4-5	121
TRPM6	Hs01019356_m1	10-17	88
RACK1	Hs00272002_m1	1-2	66
WNK1	Hs01013332_m1	2-3, 7-8	65
CUL3	Hs00180183_m1	1-2, 8-12, 14-16	67

With the primers chosen, qPCR assays were set up in DNase/RNase free 96-well plates in triplicates of each sample plated. Controls included Kidney embryonic RNA (positive), RT negative and No template control. All 3 controls were included in each plate setup. Each well-contained sample and Mastermix-primer as described in Table 2-10.

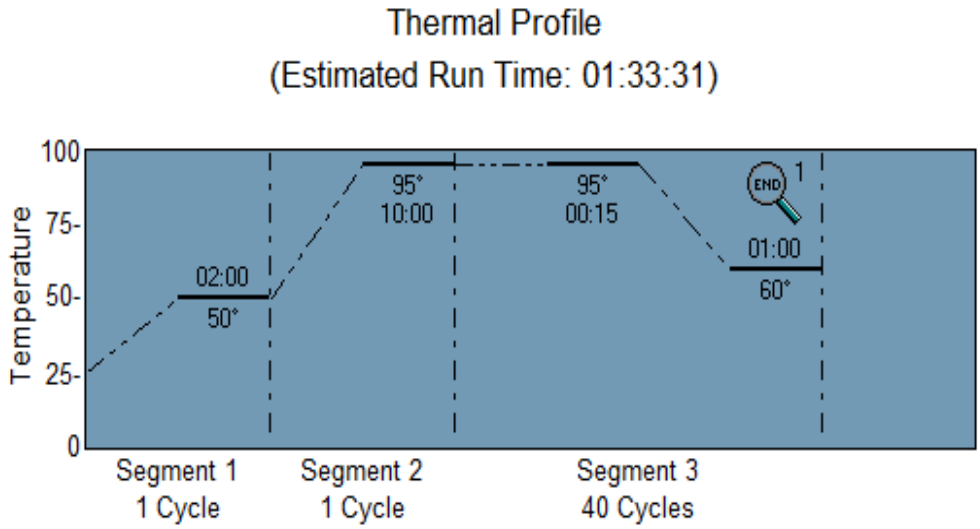
Table 2-10 Reagents required per sample per well for qPCR. *Primers and Taqman Mastermix were made into a primer/TaqMan Mastermix. This mixture was made in a separate Eppendorf tube to a volume depending on the samples and aliquoted into wells.*

Reagents	Volume (µL)
Taqman Mastermix	10
Primers	1
cDNA	9

Once plated, the plates were placed in qPCR machines, using MxPro Mx3005P to run and collected the data. FAM and ROX were used as reference dyes.

Figure 2-1 shows the time and change in temperature recommended by Taqman® Mastermix, with 40 cycles lasting 1hour and 40minutes.

Figure 2-1 qPCR cycling procedure. *The cycle describes the time lasting at each temperature. After the initial 12 minutes of a qPCR run, the denaturation (95˚C) and annealing (60˚C) was repeated for 40 cycles*



2.7.3 Analysis of qPCR using Taqman

Analysis of the data was undertaken using MxPro Mx3005P v4.01 software and Microsoft Excel each qPCR, resulting in graphs and text files. ROX was set as the reference dye and fluorescence was set to baseline (dRn). The Ct threshold was altered after each run to lie just above the background reads. The ΔCt was calculated using Microsoft Excel from the exported data files from MxPro v4.01.

2.8 RNA Sequencing

RNA sequencing (RNA-Seq) is an upcoming bioinformatics technique, using Next Generation Sequencing (NGS). Using the validation data from Nanodrop, Ribogreen, and Bioanalyzer, 10 samples were selected and standardised to 40µL in a 96-well plate. The samples were stored at -80°C and processed by Oxford Genomics (Welcome Trust, Oxford University) for further validation and sequencing.

2.8.1 RNA concentration

Due to the nature of mRNA in urinary exosomes, mRNA concentrations were low in the samples despite optimisation of methodology. Therefore, samples were concentrated using AMPure XP beads (Beckman Coulter) at Oxford Genomics [127], before commencing the library prep and sequencing. Figure 2-2 describes the process of concentrating mRNA using magnetic beads.

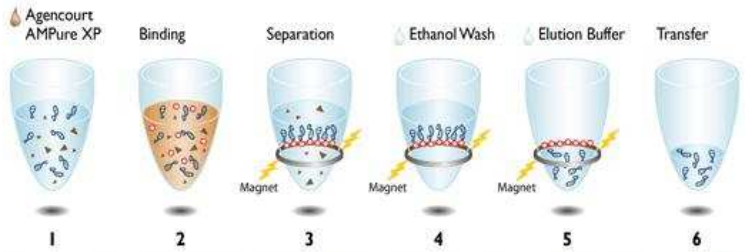


Figure 2-2 The purification of RNA sample using magnetic beads. *There are 6 stages to purifying and concentrating an RNA sample: 1) AMPure XP was added to the sample. 2) RNA fragments were bound to the paramagnetic beads. 3) A magnet was applied to separate the beads/RNA complex from contaminants. 4) The beads/RNA complex was washed twice with 70% ethanol, removing contaminants. 5) The RNA was eluted from the magnetic beads. 6) The purified sample was transferred to a new plate [127].*

2.8.2 Library quantification and sequencing of exosomal RNA

After sample concentration, Oxford Genomics (Wellcome Trust, Oxford University) performed their own quality control (QC): 9-bit, Ribogreen and Agilent Tape-Station. mRNA concentrations were not as high as anticipated, leading to one of the 10 samples failing Oxford Genomics QC and being removed from further experiments. Samples that did not degrade continued to library preparation, where ligated mRNA converts to cDNA by Reverse Transcriptase by primers targeting the 3' and 5' ends of mRNA. Agarose gel PCR showed any contamination of adapter duplexes

Figure 2-4 shows the process of library preparation. A previous decision had been made between Oxford Genomics and the group to run samples on Illumina HiSeq2500 Rapid Mode platform and select fragments between 150 and 230bp with single ends (only sequencing 5') due to the poor RIN score and low mRNA concentration. Figure 2-4 shows the attached of the adapters to the concentrated cDNA fragments (generated in library prep) and added to the flow cell. Within the flowcell, bridges are formed as an alternative PCR method, which in turn releases fluorescent light which was detected within the MiSeq® machine. At the point of quotation, 30 million reads were recommended and requested.

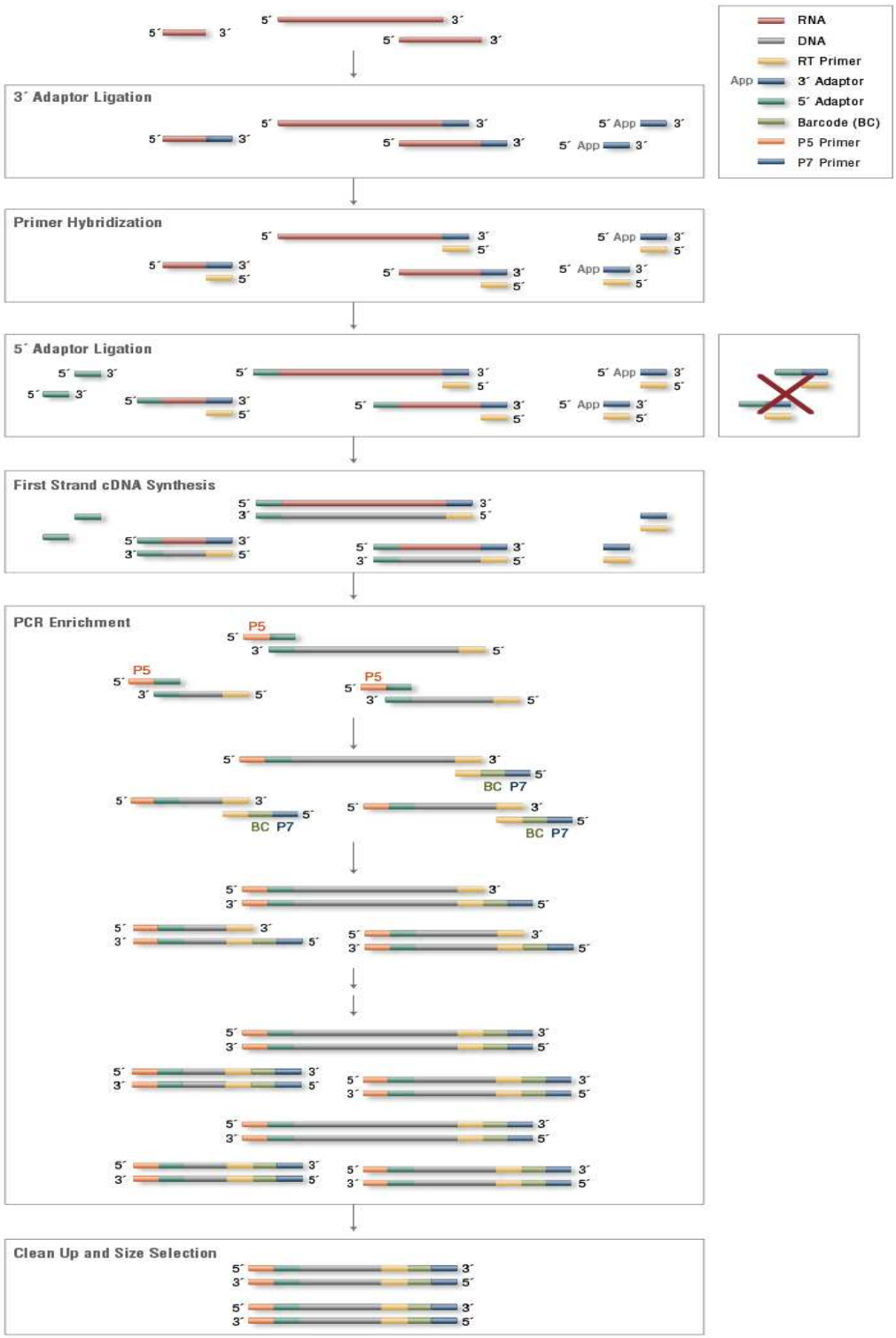


Figure 2-3 Summary of Library prep of RNA samples before sequencing. *Library preparation includes the addition of adapters and the synthesis of cDNA. In order to prepare RNA samples for sequencing, RNA must have adapters ligated to the 3' end first, before primers can attach. This was followed by the ligation of the 5' adapters. The RNA samples were converted to cDNA and enriched through PCR. The samples were cleaned of contaminants from ligation and enrichment stages before the size of cDNA fragments were selected [128].*

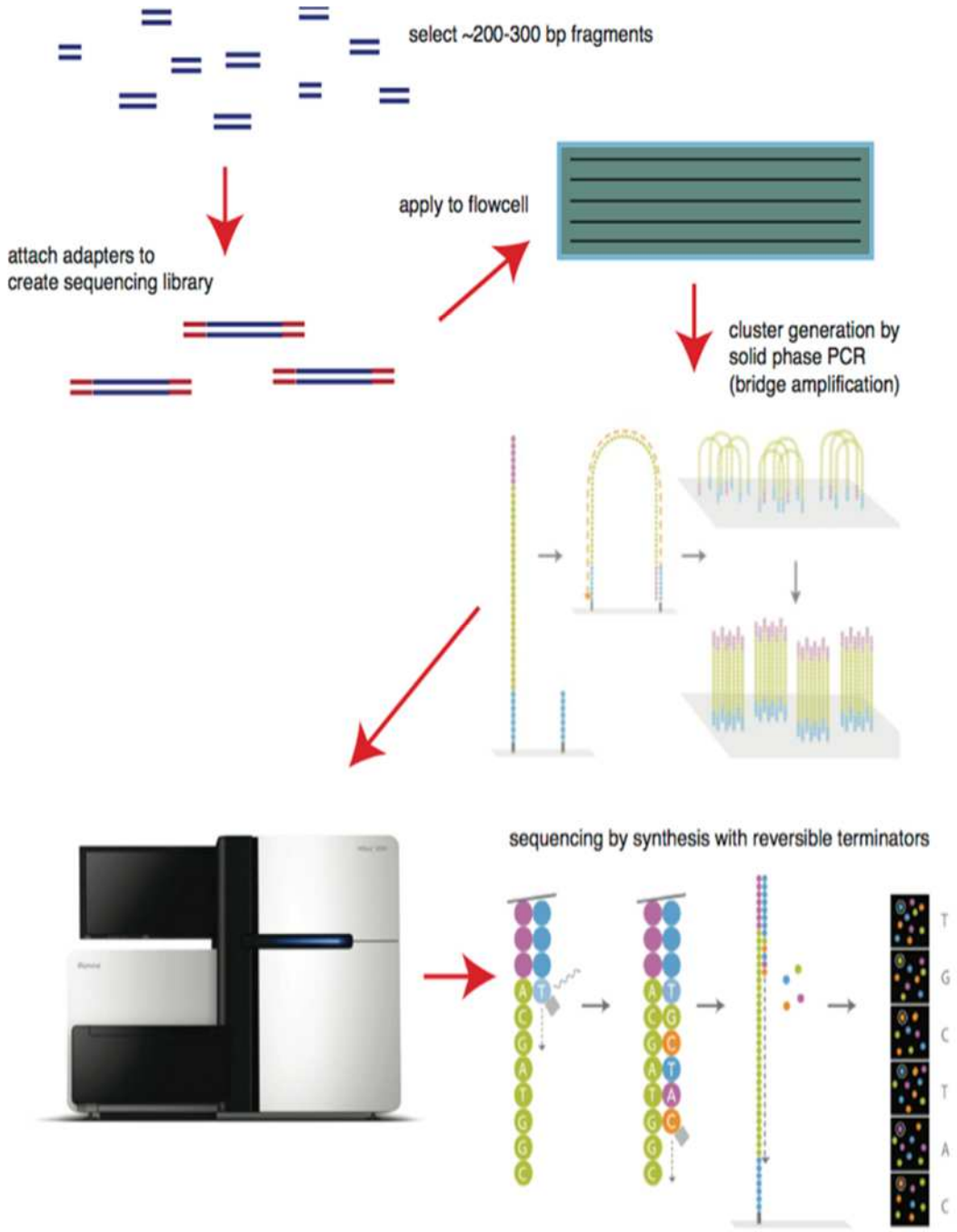


Figure 2-4 Summary of RNA sequencing after library prep. *Once library preparation was complete, fragments were selected dependent on length. In this experiment, cDNA fragments were between 150 to 250bp. Samples were added to a glass flow cell, where the sequencing reactions occurs. Adapters on the cDNA sample bind to the complementary adapters on the flowcell. A complementary strand was created using DNA polymerase. cDNA forms clonal clusters through solid phase PCR, which emit characteristic light after each cycle. Once clonal amplification was complete, the reverse strands are removed, leaving only the forward strands attached to the flow cell. Sequencing begins using the forward strands, where fluorescently labelled nucleotides bind to their complementary base. The number of cycles determines the length of reads per sample [128].*

2.8.3 Analysis of sequencing data

All analyses were performed using the Ubuntu UNIX (Linux-like) operating system version 12.04 LTS. RNA-seq output was sent in FastQ format from Oxford Genomics. Figure 2-5 shows the RNA-seq analysis protocol, starting with quality control checks. This vital for accurate analysis as low-quality nucleotides (identified by the Phred (Q) score) can affect downstream analysis. These regions of low-quality nucleotides can be identified and removed.

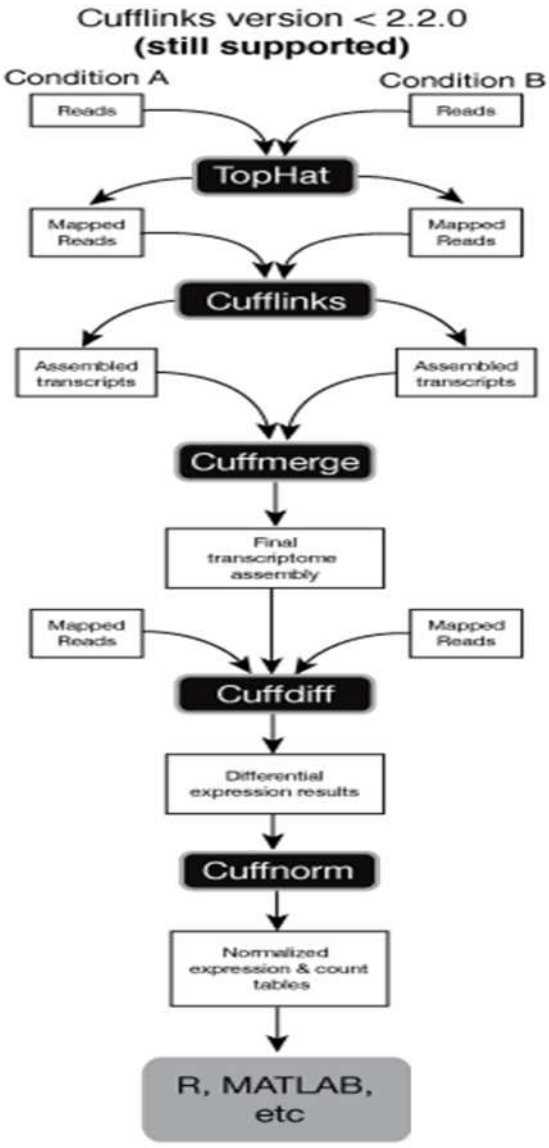


Figure 2-5 Flowchart of Cufflinks package v2.2.0. *After collating Fastq files and removing adapters using scythe and sickle programmes, RNA files were aligned with the Human Genome (v37) and merged to create transcripts. Once a merged file was created, programmes Cuffdiff and Cuffnorm were used to generate FPKM numbers. Cuffdiff compares FPKM values between different conditions, whilst Cuffnorm generates the FPKM in each sample [129].*

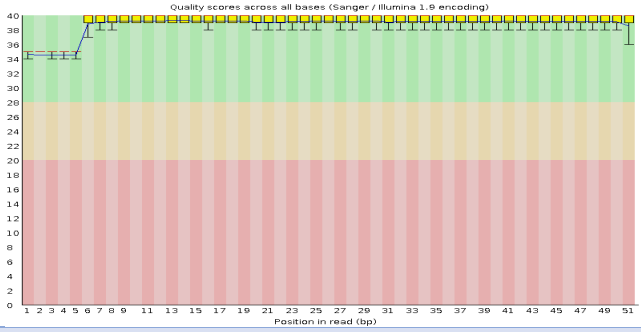
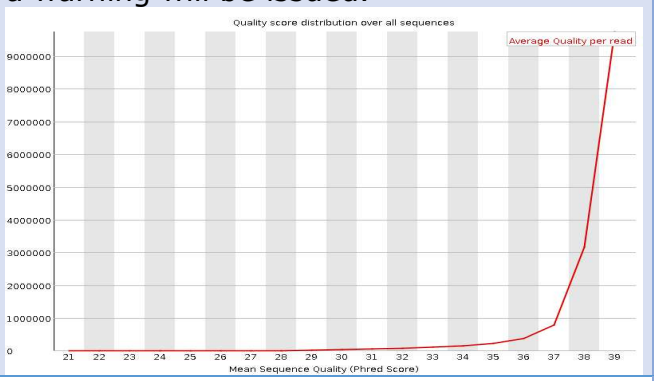
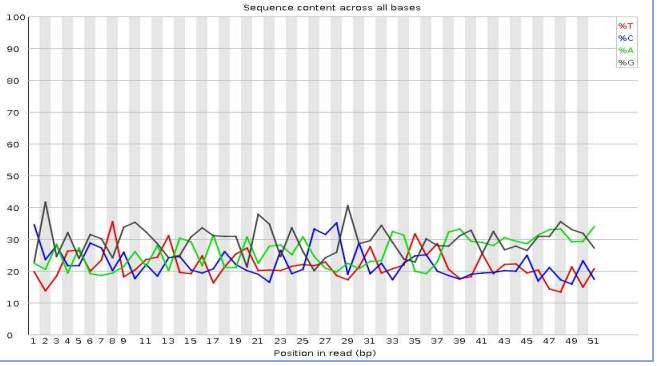
2.8.3.1 Concatenation of Data

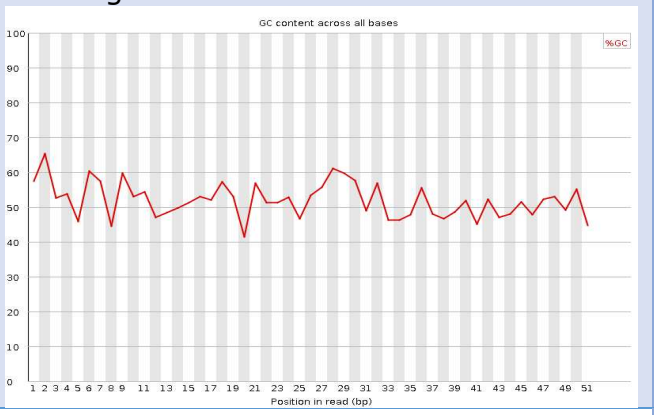
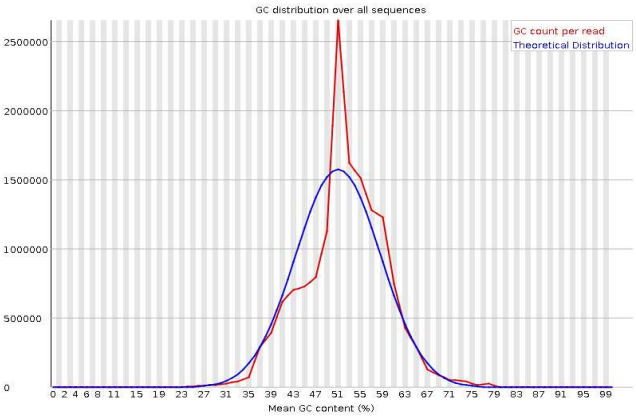
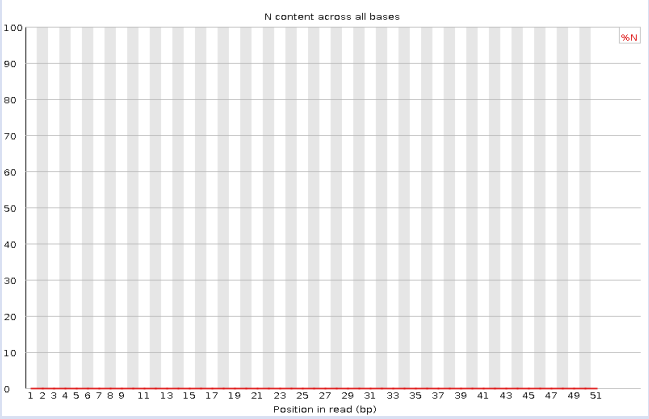
Multiple files (forward and reverse or multiple lanes) of the same sample must be combined together using the zcat concatenation command in Linux (Table 2-12). In the event of forward and reverse files, the zcat command would combine the two files to one file. FastQC was repeated to ensure no changes in the quality of reads.

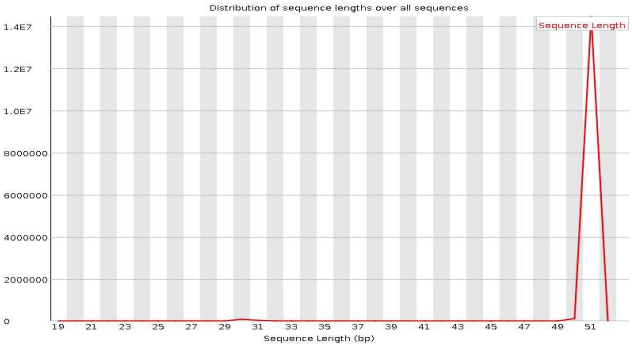
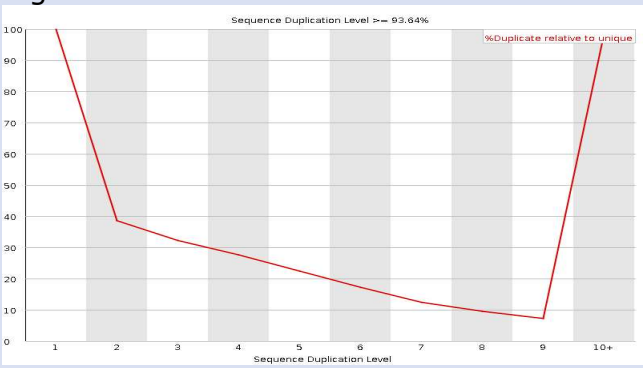
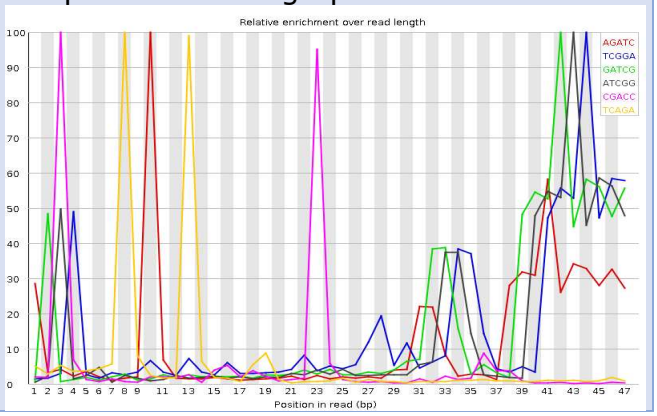
2.8.3.2 Adapter, Quality Trimming and FastQC Analysis

Adapters that were added before applying to the flowcell must be removed through "Adapter trimming". This leaves only the sequences of interest. The Scythe® tool (https://github.com/vsbuffalo/scythe). To read the Q score, "Quality Trimming" was performed using Sickle (https://github.com/najoshi/sickle). FastQC identifies any issues, which may affect any further analysis. This tool can locate where any issues originate e.g. within the sequencer or the original sample. Table 2-11 shows each module produced by the FastQC tool and their individual functions.

Table 2-11 Details of each FastQC output. An example is taken from Chapter 5.

FastQC module	Function
Basic Stats	Includes Total number of sequences processed, sequence length, overall %GC of all bases in all sequences
Per Base Sequence Quality	Displays the quality across all bases. Green = good quality, yellow = average quality, red = poor quality. If the lower quartile of a base was less than 10, or the median was less than 25, a warning will be issued. 
Per Sequence Quality Scores	Identifies any low-quality values across sequence fragments. If the mean sequence quality was below 27 (or 0.2% error rate), a warning will be issued. 
Per Base Sequence Content	Displays the base position proportion in each sample, in the order each of the four base-pairs has been called. If the bases differ more than 10% between A and T or G and C, a warning was issued. 

Per Base GC Content	The GC content was measured in each sample. If the GC base deviates from the GC content mean by more than 5%, a warning will be issued. 
Per Sequence GC Content	GC content was measured throughout the sequence, and compared to a theoretical distribution. A deviation of more than 15% will trigger a warning in the module. 
Per Base N Content	Displays percentage of bases that weren't called by the sequencer (N). If the N content was greater than 5%, a warning will be issued. 

Sequence Length Distribution	Fragments sizes are displayed in this module. A warning was generated if sequences are not equal lengths. 
Duplicate Sequences	This module plots the degree of duplication in every sequence within each sample. A low duplication number might suggest a high level of coverage. A warning was issued if the degree of duplication was higher than 20%. 
Overrepresented Sequences	All sequences that make up more than 0.1% of the total are listed in this module. Sequences repeated many times could indicate a biologically significant sequence, contamination or the sample was not as varied.
Overrepresented Kmers	Every k-mer was counted and calculated within the sequence. Only the 6 top k-mers are plotted on the graph 

2.8.3.3 Tophat2 Alignment

Using the TopHat2, the spliced fragments of RNA are aligned to the reference human genome (GRCh37, http://grch37.ensembl.org/index.html) using the Bowtie command line. Bowtie was a high-throughput short read alignment tool (http://bowtie-bio.sourceforge.net), which produces BAM files from the previous concatenated RNA sample files. With these samples now aligned to the referenced genome, the set-up of each sample was complete and analysis can begin.

2.8.3.4 Differential Gene Expression Analysis

Cuffdiff is one of several commands included in the Cufflinks software package (v2.2.1) [130] and is used to compare gene expression between samples. To achieve this analysis, Cufflinks and Cuffmerge were used to produce and make a "master transcriptome", respectively. See Table 2-12 for detailed command lines. The aligned "accepted_hits.bam" files are used to compare between each other, producing Fragments Per Kilobase of transcript (FPKM) along with statistical significance. The output files from each Cuffdiff command produces text files, which can be viewed in Microsoft Excel.

2.8.3.5 Using Cuffnorm to determine gene expression

The command line for Cuffnorm produces FPKM counts, with the larger number considered as highly expressed in the sample. It was decided to use a FPKM number of 10 or higher as a cut off based on previous experience.

2.8.3.6 Command lines and codes

Table 2-12 describes all the command lines and their functions used through RNA analysis. Sanger platform was selected by the Oxford Genomics company, and therefore part of the parameters and essential to use in the command lines below.

Table 2-12 List of command, their functions and command lines.

Command	Function	Command line
Zcat	Concatenates Data	zcat *(samplename)* | wc -1
Sickle	Removes low quality reads	path/sickle se –f *(samplename)*.fastq –r *(samplename)*.fastq –p qtrim.2.fastq –s qtrim.unpaired.fastq –q 20 –l 20
Scythe	Removes adapter sequences	path/scythe –a adapter_file.fastq –q sanger –p 0.1 –o *trimmed(samplename)*.fastq sequence.fastq
FastQC	Presents quality controls of data	fastqc path/fastq
Read alignments	SAM/BAM produces a list of read alignments	accepted_hits.bam
Cufflinks	Assembly and quantification of transcriptome	cufflinks –p 2 –o *(samplename).*accepted_hits.bam
Cuffmerge	Merges all sample transcripts into a master transcriptome	cuffmerge –g *(location of Human Genome 37).*gtf –s *(location of Human Genome 37).*fa –p 10 assemblies.txt
Cuffdiff	Compares gene expression between samples.	Cuffdiff –o diff_out *(location of newly created file)*.gtf –p 10 *(location of the file to compare),(location of the file to compare)*
Cuffnorm	Presents and normalises FPKM values	Cuffnorm –use sample sheet *(location of merged file).*gtf –p 10 *(location of sample sheet)*

Due to the novel circumstances of sequencing exosomal RNA, all RNA samples were read as a single read file, along the sense strand. Quality of the exosomal RNA was too low to consider paired-end reads.

Table 2-13 Codes used to within command lines. These codes describe functions or dictate threshold.

Codes	Functions
p	Determines the number of threads used to align reads
q	Defines the Phred score and determines the quality cut-off for trimming output file.
o	Determines the output file
t	Determines the quality scheme in the platform used (Illumina/Sanger)
M	Only used if FastQ file did not pass the filter reads
se	Single end
f	Determines the forward read file
G	Provides a set of gene models for TopHat

2.9 Cell culture preparation

2.9.1 General methods

Cells were thawed from liquid nitrogen. All reagents were heated to 37°C before using within cell culture. All four cell lines were cultured using Cell culture Media (from now called Complete Media). The cells lines included in experiments were:

- HEK-293 (Wild-Type)
- HEK-NCC (a transfected cell line which overexpresses NCC)
- HEK-EV (transfected empty-vector control for HEK-NCC)
- PKTC (to measure the biological effect)

Table 2-14 describes the individual reagents added to the complete media. 15mL of complete media was added to each T75 flask. HEK-NCC and HEK-EV cell lines were generated by Prof D. Alessi's group, University of Dundee.

Table 2-14 Reagents to make Complete Media

HEK-293	HEK-NCC and HEK-EV,	Proximal Kidney Tubule Cells
Dulbecco's Modified Eagle's Medium (DMEM)	Dulbecco's Modified Eagle's Medium (DMEM)	Renal Epithelial Cell Basal Medium
Fetal Bovine Serum (10%)	Fetal Bovine Serum (10%)	Fetal Bovine Serum (0.5%)
Penicillin (100units/ml)	Penicillin (100units/ml)	Penicillin (100units/ml)
Streptomycin (0.1mg/ml)	Streptomycin (0.1mg/ml)	Streptomycin (0.1mg/ml)
	Blastocidin (15µg/ml)	Triiodothyronine (10 nM)
	Hygromycin (0.1mg/ml)	Rh EGF (10ng/mL)
	Tetrocycline (1µg/ml)	Hydrocortisone Hemisuccinate (100ng/mL)
		Rh Insulin (5µg/ml)
		Epinephrine (1.0µM)
		Transferrin (5µg/ml)
		L-Alanyl-L-Glutamine (2.4mM)

After recovery of cells, each flask was left for 24hours for cells to adhere, before removing the residues of the freezing mixture (90% FBS+10% DMSO). Passaging cells involved adding 5mL of trypsin and incubating at 37°C for 5 minutes. Once the cells detached, 10mL of Complete Media was added to inactivate the trypsin. The cells were removed from T75 flasks into 50mL falcon tubes and centrifuged at 200g for 5mins. Cell pellets were re-suspended with 1mL Complete Media. This was followed by seeding cells back to fresh T75 flasks with fresh Complete Media.

Before cells were seeded to 6 well plates, the cells were split at between 90-100% confluency and haemocytometer used to count cells. In each well of a 6-well plate, 300,000 cells were seeded in exosomal-depleted media and incubated for up to 2 days for 100% confluency. Media samples were collected every 24hours and stored at -80°C with protease inhibitor. With HEK-NCC and HEK-EV cell lines, 1µg/mL of tetracycline was also added to induce expression.

2.9.2 Transformation and Transfection of HEK-293 cells with a pcDNA5/FRT Empty Vector

Although the following methods describe the process of transformation/transfection of HEK-293 cells, the resulting HEK-EV cell line was generated by Prof. D. Alessi's group, University of Dundee. Attempts were made within the laboratory, but transfections failed continuously.

2.9.2.1 Transformation

Transformation experiments were carried out to make an empty-vector HEK-293 cell line, as a control for HEK-NCC. In order to "bulk up" the pcDNA5/FRT (Invitrogen) plasmid was transfected into Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen).

Cells were placed on ice after recovering from liquid nitrogen. Once thawed, 50uL of cells were aliquoted into 1.5mL tubes. The vial of pcDNA5/FRT plasmid and pUC19 (positive control) (Invitrogen) was thawed, before adding 5uL to the cells in the 1.5mL tube. The pcDNA5/FRT and cells were mixed gently. Using the heat-shock method, the cell mix was incubated on ice for 30 minutes, before moving the cells to a 42°C water for 20 seconds. The tubes were then placed back on ice for 2 minutes.

After finishing the heat-shock method, 250uL pre-warmed Complete Media was added to the newly heat-shocked cells. The tubes containing the cells were incubated at 37°C for 1hour in a shaker. Each transformation was spread on pre-warmed selective LB agar plates including 100ug/mL ampicillin. The plates were incubated overnight at 37°C.

2.9.2.2 Mini-prep and sequencing

To extract and purify the DNA, the Wizard®Plus SV Minipreps DNA Purification System (Promega) was used. After the selection of the positive bacterial colonies, they were pelleted for 5minutes. The pellet was re-suspended in 250uL of Cell Resuspension Solution, before inverting for 4 times to thoroughly mix. To lyse the cells, 10µL of Alkaline Protease was added and inverted 4 times. The lysed cells were then incubated at room temperature for 5minutes. A Neutralisation Solution (350uL) was used and again inverted 4 times to mix. The tube was centrifuged at 13,000rpm for 10minutes at RTe.

The lysate was loaded onto a spin column and centrifuged at 13,000rpm for 1minute at RTe. The flowthrough was discarded, before adding Wash Solution (750uL with the first spin, 250uL for the second spin), (ethanol must be added to this solution before starting experiment). The tube was centrifuged at 13,000rpm for 1 minute with the flowthrough discarded once the spin was complete and repeating this again. The tubes were spun again for a further 2 minutes at RTe.

The spin columns were then transferred to sterile 1.5mL tubes, where after 100uL Nuclease-free water to the spin column. The tubes were centrifuged at 13,000rpm for 1 minute at room temperature. Samples were stored at -20°C until required for sequencing.

2.9.2.3 Maxi-prep

A starter bacterial culture was incubated in 200mL LB broth (includes 100ng/µL ampicillin) overnight at 37°C in a shaker (approximate 300rpm). The culture was harvested by centrifuging at 6000g for 15 minutes at 4 °C. Using the Qiagen Maxi Prep kit, the supernatant was decanted and the pellet is re-suspended in 10mL of lysis buffer (Buffer P1) making sure of complete suspension. An additional 10mL of lysis buffer (Buffer P2) was added and

vigorously shaken before incubated at RTe for 5 minutes. The precipitation buffer (Buffer P3) was added (10mL) and mixed thoroughly until the white fluffy materials were observed. The mixture was placed on ice for 20 minutes. The mixture was centrifuged at 20,000g for 30 minutes at 4 °C. The supernatant created contained the required plasmid DNA. The supernatant was centrifuged again at 20,000g for 15 minutes at 4 °C and the new supernatant was removed again. This supernatant was added to a QIAGEN-tip and left to be emptied by gravitational flow. Once all supernatant passed through the QIAGEN-tip, 2x 30mL of a wash buffer (Buffer QC) was added and allowed to pass through the filter tip. The DNA was eluted from 15mL of elution buffer (Buffer QF).

In order to clean the DNA, 10.5mL (0.7 of the DNA volume) of isopropanol was added, mixed, and centrifuged at 15,000g for 30 minutes at 4 °C. The supernatant was carefully removed and the DNA pellet was washed with 70% ethanol, before centrifuging at 15,000g for 10 minutes. The pellet remaining was not to be disturbed by the supernatant that was removed. The DNA pellet that remained was air-dried for 5-10 minutes. Once dried, the DNA pellet was reconstituted in 50µL of TE elution buffer. The concentration of the dissolved DNA was determined using the Nanodrop®.

2.9.2.4 Transfection

As this experiment was to create a HEK empty vector control, no insert DNA was included. All reagents were added together and gently mixed and centrifuged at maximum speed. The tube was incubated for 1 hour at 37°C. Table 2-15 details the reagents required for the linearization of the plasmid.

Table 2-15 Reagents for use of BamH1 with the pcDNA5. *A Mastermix was created in one tube. A negative control, with no enzyme, was also made alongside the DNA sample. Both tubes were used in the gel.*

Components	Volume (uL)
Sterile, nuclease-free water	15.8
Restriction Enzyme 10X buffer	2
Acetylated BSA (10ug/uL)	0.2
pcDNA5/FRT (1ug/uL)	1
BamH1 (restriction enzyme)	1

The samples were loaded on a 1% agarose gel and run at 35V for 30 minutes. The gel was viewed using the Gel Genius geldoc, to confirm the linearization of the plasmid.

At the point of transfection, HEK 293 cells were seeded in a 6-well plate. After 18-24 hours to allow for adherence, a chemical transfection mix was produced (Table 2-16).

Table 2-16 List of reagents used to make Transfection mix in 1 well from a 6-well plate

Components	Volume
Complete growth medium	2.5mL
Serum-free medium	150uL
DNA (1ug/ul)	100uL
***Trans*IT-LT1 Reagent**	7.5uL

The reagents were mixed gently and incubated at RTe for 30 minutes. The transfection mix was added dropwise to each well and rocked gently for even mixture. The cells were incubated for 48 hours without changing the media. After the 48 hours, the cells were passaged and seeded into Complete Growth Media, which includes Hygromycin B for stable selection. Selection using Hygromycin B continues for 2 weeks to allow for stable integration of plasmid DNA.

2.9.3 Collection of exosome rich media

Cell lines, HEK (wild-type), HEK-EV (empty pcDNA5 vector), HEK-NCC (pcDNA5 vector with FLAG-NCC insert), and Primary epithelial Proximal Kidney Tubule Cells (PKTC), were passaged and seeded into 6-well plates or T75 flasks. One 6-well plate/T75 flask was used per cell line. For cells were seeded into 6-well plates, $0.3x10^6$ cells were plated per well, whilst $2.5x10^5$ cells were seeded into T75 flasks.

For cells that were growing in the 6-well plates, exosome-free media replaced the original media (for usual cell culture) after 24hours. Each cell line was incubated for a further 24 hours before the media is collected and frozen at -80°C. The cells are also trypsinized and frozen for protein work.

For cells growing in T75 flasks, exosome-free media replaced the original media after 24 hours. The cells were allowed to reach 70-80% confluency before changing the media and collecting media supernatant 24 hours and 48 hours later.

2.9.4 Harvesting cells for protein work

In 6-well plates, cells were harvested after 48 hours of incubation. A protein lysis buffer was added to the top 3 wells and cells were scrapped into the buffer using a cell scraper. The cell/protein lysis buffer was transferred to the lower 3 wells and the process was repeated. Once all cells had been removed into the lysis buffer, the solution was transferred to a 1.5mL tube and stored at -80°C.

Cells growing in T75 flasks were trypsinized and a cell count was performed for each flask. The cells were spun down again and 1mL of protein lysis buffer was added to the cell pellet. The lysate was transferred to a 1.5mL Eppendorf tube and stored at -80°C.

Table 2-17 Reagents for Protein lysis buffer used to harvest cells. *The mixture was made prior to harvesting cells. 1mL was used per T75 flask. 300µL was used per 2 wells when harvesting cells from 6-well plates.*

Reagents	Volume
Cytobuster buffer cOmplete Mini Protease Inhibitor in Cytobuster Protein extraction Reagent (Millipore)	1mL
Benzonase nuclease (Millipore)	10µL
DTT	1 µL
Pepstatin A (0.5mg/mL)	2µL

2.9.5 Labelling Cells and Exosomes

PKTC cells were trypsinized and pelleted before a 10nM CellTracker 565 (green) (Invitrogen) labelling solution was made. The mixture was incubated for 5 minutes at RTe, before vortexing vigorously for 30 seconds. The labelling solution was incubated for 1 hour at 37°C at 5% CO_2. The cells were washed in complete growth media, before seeding into a T75 flask. After 24 hours after, the PKTC cells were visualised before adding the exosomes (Section 2.9.7).

The exosome fraction was extracted on the day of stimulation, before incubating with QTracker 655 (red) (Invitrogen). The labelling of the exosomes was the same as above. After incubation with the QTracker, the exosomal pellet was washed using Vivaspin™ tubes with exosome-depleted Complete Media and added to PKTC cells.

2.9.6 Imaging HEK and PKTC cells

PKTC and labelled HEK cells were imaged using the Olympus Inverted Microscope CKX53 and Olympus camera XM10. PKTC cells were imaged at 40x magnification using brightfield, blue and green filters on the Olympus Inverted Microscope CKX53.

2.9.7 Exosomal NCC exchange to PKTC cells

Once exosome-rich media from HEK, HEK-EV and HEK-NCC had been collected, extracted and labelled as described in sections 2.9.3 and 2.9.4, the exosomes were added to PKTC cells (cell confluency=70-80%) in T75 flasks. The plates were incubated in the exosome fractions for 24 hours. Pictures were taken of the exosome-PKTC combination using the Olympus Inverted Microscope CKX53 and Olympus camera XM10, before and after 24 hours.

2.10 Statistical Analysis

A range of statistical approaches were used as appropriate for each separate analysis.

2.10.1 Power calculation

A Power calculation was carried out to identify the number of subjects required for correlations presented in Chapter 4. As the primary analysis was to compute Pearson's correlation for the associate between 2 continuous variables, Nquery Advisor A was used to generate a sample size by inputting three variables:

- Power=80%
- Effect size=0.5 (50%) (50% increase or decrease in protein expression)
- Significance=0.05

The resulting calculation performed by Nquery Advisor showed that to achieve the desired power and effect, 30 samples were required for experiments in Chapter 4.

No other power calculations were performed for experiments carried out in Chapters 3, 5 & 6. Sample sizes were selected based on economic reasons.

2.10.2 Tests of Normality

To test the distribution of the data collected, Normality tests were performed. As sample sizes were small throughout all experiments, it is assumed that they are not powered enough to give the typical Gaussian distribution. Therefore, parametric tests were performed to measure for normality, where any p-value of <0.05 was assumed as a non-parametric distribution.

2.10.3 Linear Regression

To measure the relationship strength between two variables, linear regression was performed, for example when comparing protein expression and electrolyte concentration. A straight line represents the best fit between the associated data, producing an equation of the Y-intercept and change in X.

2.10.4 Correlation Analysis

Correlation analysis uses linear regression to identify the correlation coefficient (r), determining the relationship between two variables through numerical figures. Where r=+1, it is assumed that the correlation is strong and positive, however where r=-1, the correlation is assumed strong, but negative. Any r values close to equalling 0, this is interpreted as a weak or no correlation.

2.10.5 One-way Anova

Using GraphPad Prism V6.05, the average±SEM of the expression of each protein was reported and presented by graphs. (ANOVA) were performed to identify any significant difference in expression between each protein, where $p<0.05$ was considered as significant.

Chapter 3: Validating the use of urinary exosomes for western blotting and the potential role of exosomes in extracellular communication

3 Validating the use of urinary exosomes for western blotting and its role in extracellular communication

Abstract

Extracellular Vesicles (EV) are membrane-bound bodies ranging from microvesicles, to apoptotic bodies, to exosomes. Exosomes are an excellent resource for providing insightful views of the inner mechanisms of cells. The cell's proteins, DNA and RNA are found inside exosomes which originate from the cytoplasm of the parent cell. Specific biomarkers can be used to identify the exosomes origin, therefore providing the potential to study specific tissue without the need for a tissue biopsy.

The functionality of exosomes is still uncertain, however, there is increasing interest in their potential role in cell-to-cell communications.

Using urine, the work in this chapter aims to determine the optimal conditions for urinary exosomal fraction storage and quantify the urinary exosomal fraction from the ultrafiltration extraction method. Experiments in cell culture will also explore the role of exosomes as an extracellular communication vessel between HEK 293 cell lines and Primary Epithelial Proximal Tubule Kidney cells.

Using the ultrafiltration method showed that NCC, pNCC, ALIX and AQP2 expression were found almost exclusively in the exosomal fraction when compared to the cell pellet and flow-through fractions. The protein expression was not affected by storing samples in -80°C. It can be concluded from these results that the exosome fraction can be used to quantify NCC and related protein expression.

Cellular experiments aimed to show exosomal NCC can be transferred to PKTC to add to the growing evidence that exosomes can be used for cell-cell communication. Whilst initial results showed that NCC had transferred to PKTC, there was no difference in expression in NCC or FLAG-NCC between the 3 PKTC cell populations stimulated by the 3 different NCC-containing HEK

exosomes. Without repeated and further investigations, it is difficult to conclude whether there was an experimental error or that PKTC expression of NCC is not dose-dependent. Further experiments should include confocal microscopy to identify if the exosomes have entered the PKTC cells and radiology experiments to identify the functionality of NCC.

Overall, this chapter has validated the use of exosomes for protein quantification in urine and cellular media. The method of extraction will be applied in the remainder of the results chapters of this thesis.

3.1 Introduction

As discussed in the first chapter of this thesis, exosomes, first discovered 30 years ago, are small microvesicles released from a variety of eukaryotic cells i.e. immature red blood cells, white blood cells, and epithelial cells [131, 132]. Because of their origin, exosomes provide an insightful view of the inner mechanisms of cells, as proteins, DNA and RNA are found inside exosomes which originate from the cytoplasm of the parent cell [133]. With this new resource, exosomes provide a new direction to researching cell biology, whilst offering alternatives to tissue biopsies, which are invasive and painful procedures. Exosomes can be collected from any biological fluid e.g. urine or blood. The use of urinary exosomes is a novel technique within research, allowing new real-time information to be collected from the bladder and the kidney, without the need for biopsies.

Exosomes are part of a subset of membrane-bound bodies, known as Extracellular Vesicles (EV). Other membrane vesicles include apoptotic bodies and microvesicles [118]. Differentiating between these different vesicles with current technology is difficult and expensive [118], however, the presence of NCC as a specific biomarker suggests the origin of these vesicles is from the distal nephron [134].

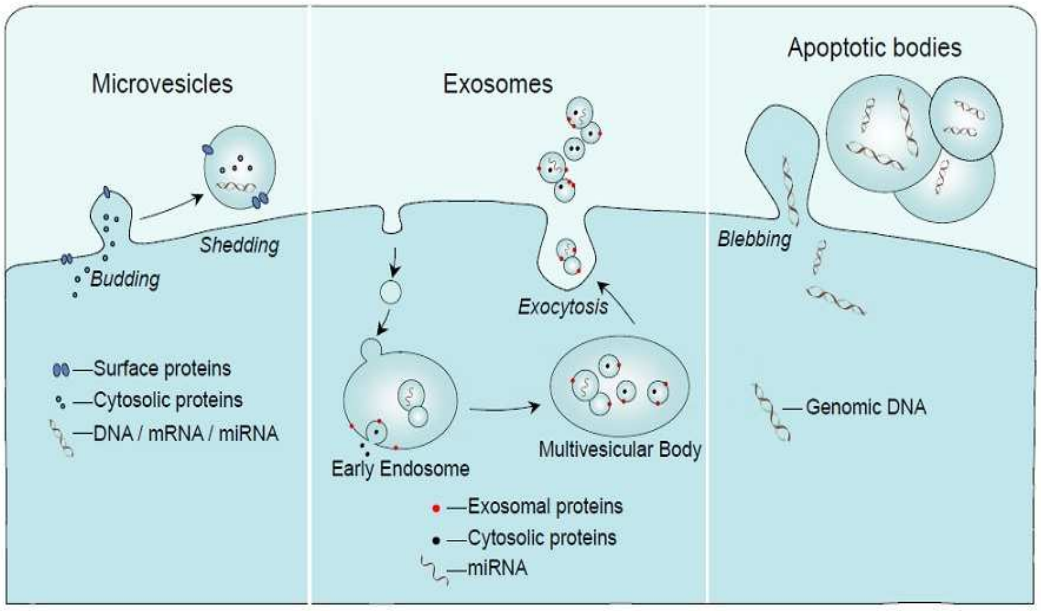


Figure 3-1 Structural differences between the Extracellular Vesicles. *The three membrane-bound vesicles contain different molecular substances. Unlike the other extracellular vesicles, exosomes contain proteins unique to itself, ideal for communicating with neighbouring cells. This also makes exosomes ideal for isolating from apoptotic bodies and microvesicles [135]***.**

One of these markers is AQP2, which is involved in water balance disorders [136], and one previous study suggested that EV containing AQP2 on the membrane can be considered as exosomes [137].

ALIX or ALG-2-interacting protein X is a regulator of cell death (apoptosis) [114]. It is also highly concentrated within exosomes, due to being heavily involved in the budding process of exosomes from the multi-vesicular bodies [138, 139]

Currently, there is no set house-keeping protein for identifying exosomal fractions similar to those available for RNA work such as B-actin. As both AQP2 and ALIX are believed to be markers only present in exosomes, these two proteins would appear to make ideal house-keeper proteins for proteomic work in exosomes. However, AQP2 may alter in conditions of electrolyte or volume plasma dysfunction and hence it is uncertain if this makes AQP2 the better candidate. There is yet to be a formal comparison and they have yet to be formally compared. As part of the work presented in this chapter, this comparison will be addressed.

Various methods have been described to extract exosomes, ranging from ultracentrifugation, ultrafiltration, and exosome binding materials (Exoquick and Total Exosome Isolation) [132, 140]. All extraction processes vary in how they are likely to be useful depending on the material to be isolated (mRNA or protein) [132, 140]. In addition, because exosomes are a relatively novel research area, there is no set protocol for extracting and storing these nano-vesicles.

The functionality of exosomes is still uncertain. However, there is increasing interest in their potential roles in cell-to-cell communications, including the transfer of genetic material, proteins and soluble factors [141]. Recently, exosomes have been used to identify certain cancer tumours e.g. prostate cancer, as well as using the vesicles as potential immunotherapy treatments [142, 143].

The work in this chapter aims to determine the optimal conditions for exosomal fraction storage and quantify the exosomal fraction from the ultrafiltration extraction method. Experiments in cell culture will also explore the role of exosomes as an extracellular communication vessel between HEK 293 cell lines and Primary Epithelial Proximal Tubule Kidney cells.

3.2 Optimal urine fractions and storage conditions for exosomes

To confirm exosomal fractions from ultrafiltration extraction, validation experiments are essential. Using exosomal markers previously described, western blots were used to confirm exosome presence. It was vital to confirm the stability of exosomal proteins once extracted. Urine samples used were collected from non-hypertensive normonatraemic controls and treated with protease inhibitor. Samples were stored at -20°C and -80°C for up to 1 month.

3.2.1 Urine Fractions

Samples from healthy volunteers were collected and stored at -80°C for up to 1 month. As Figure 3-2 shows below, exosomes highly express NCC (6.61a.u.±1.73) and AQP2 (4.09a.u.±1.06) in comparison to the cell pellet and flow through. One-way Anova was used for statistical analysis and showed a significant difference in the expression of the proteins between exosomes, cell pellets and flow through ($p<0.05$).

Minimal expression of NCC (0.35a.u.±0.12) and AQP2 (0.7a.u.±0.69) can be seen in the cell pellets, but this is much lower than that seen in exosomes. Expression in the flow through fraction shows little to no expression (NCC=0.22a.u.±0.3/AQP2=0.13a.u.±0.33).

3.2.2 Storage Conditions

Figure 3-3 shows exosomes stored at -80°C have a significantly higher expression of NCC (6.61a.u.±1.73, $p<0.05$) and AQP2 (4.1a.u.±1.6, $p<0.05$) compared to exosomes stored at -20°C. Exosome samples extracted from fresh urine (then stored at -80°C until western blot use) express NCC (3.62a.u.±1.72) and AQP2 (1.26a.u.±2.3), but not as strongly as samples stored at -80°C. However, the difference in expression was not statistically significant ($p>0.05$).

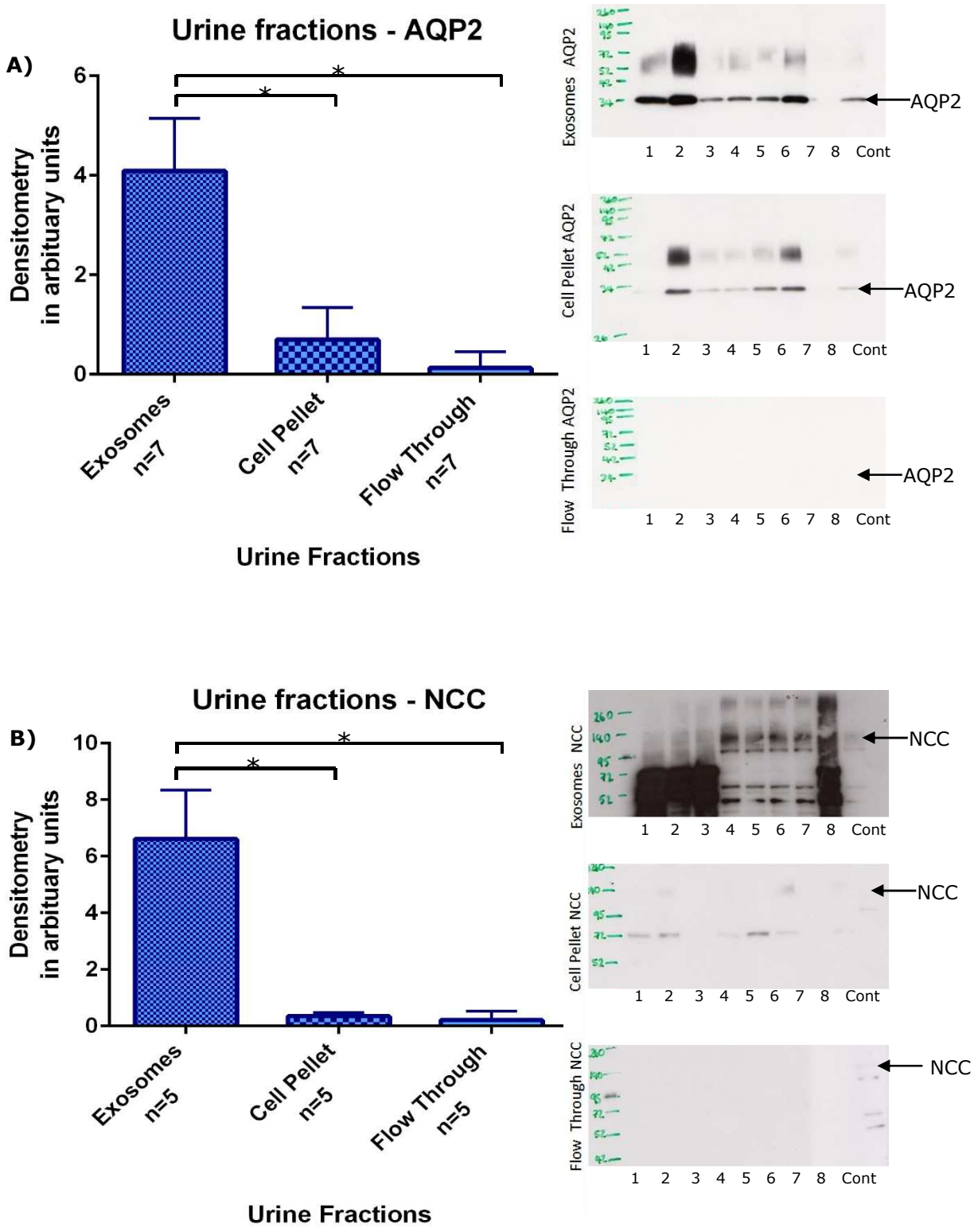


Figure 3-2 Expression of AQP2 and NCC in Urine fractions. *A) AQP2 (~34kDa) is highly expressed within the exosome compared to the Cell Pellet and the Flow Through urine fractions B) NCC (~140kDa) is highly expressed in the exosomes, compared to the cell pellet and the Flow Through urine fractions (*, P<0.05).*

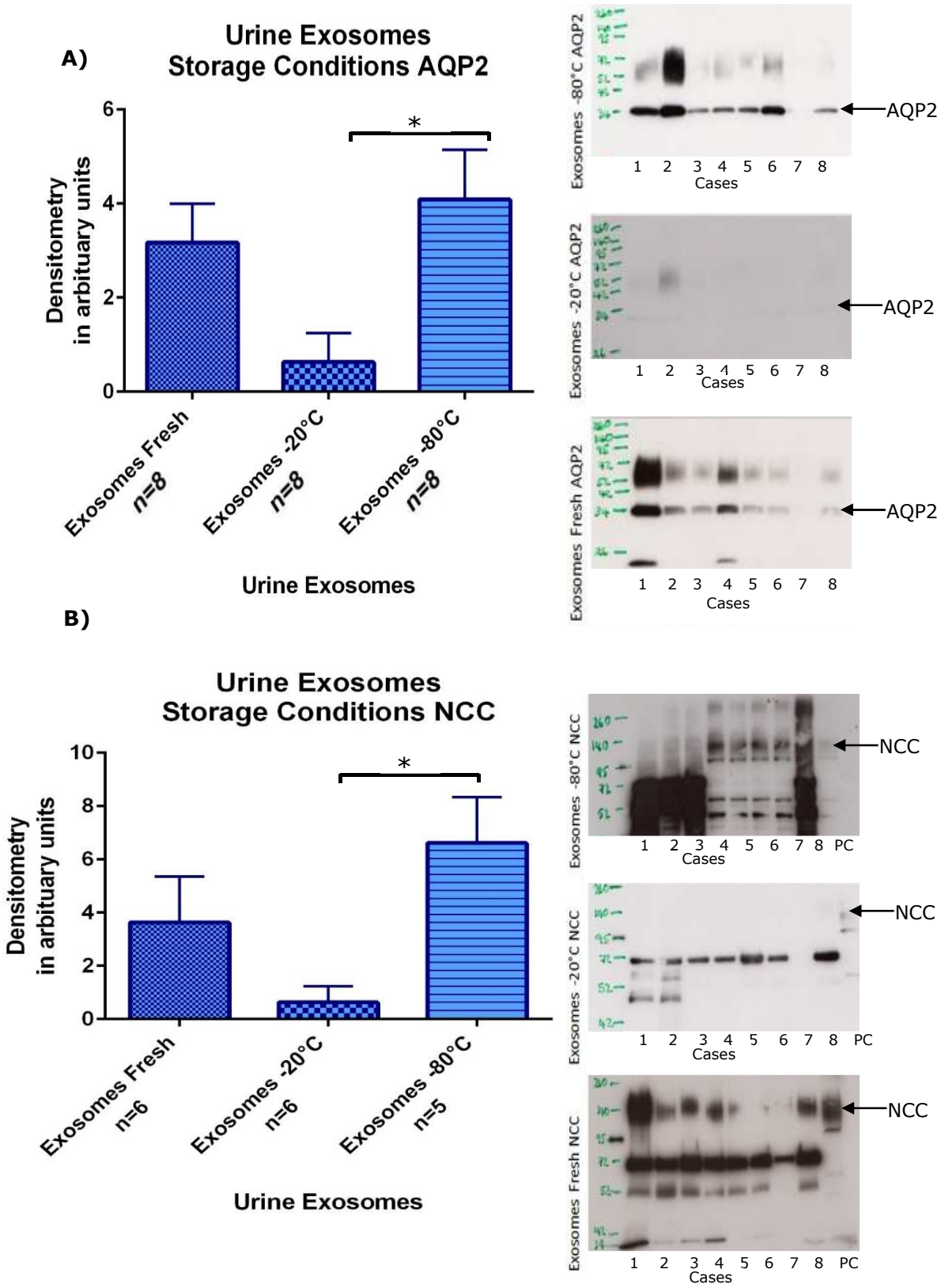


Figure 3-3 Expression of AQP2 and NCC in urine exosomes stored at various temperatures. *A) AQP2 (~34kDa) is highly expressed at the storage condition of -80°C, but some expression can be seen in exosomes extracted from fresh and when stored at -20°C. B) NCC (~140kDa) is highly expressed when stored at -80°C, with expression also seen in freshly extracted exosomes. Little to no expression was seen at -20°C. See below (*, P<0.05).*

3.2.3 The expression of phosphorylated NCC

Total NCC and the level of expression of the phosphorylated protein was determined using antibodies specific for each phosphorylation site, i.e. N-terminal phosphoryl sites (S91, T55, and T60) (Figure 3-4). Total NCC expression was higher (6.61±1.73), than all three of the phosphorylated NCC isoforms, which showed lower expression on average (P60=1.14±1.34, S91=1.14±1.96, T55=1.89±3.15). This shows that active NCC can also be detected in exosomes as well as total NCC.

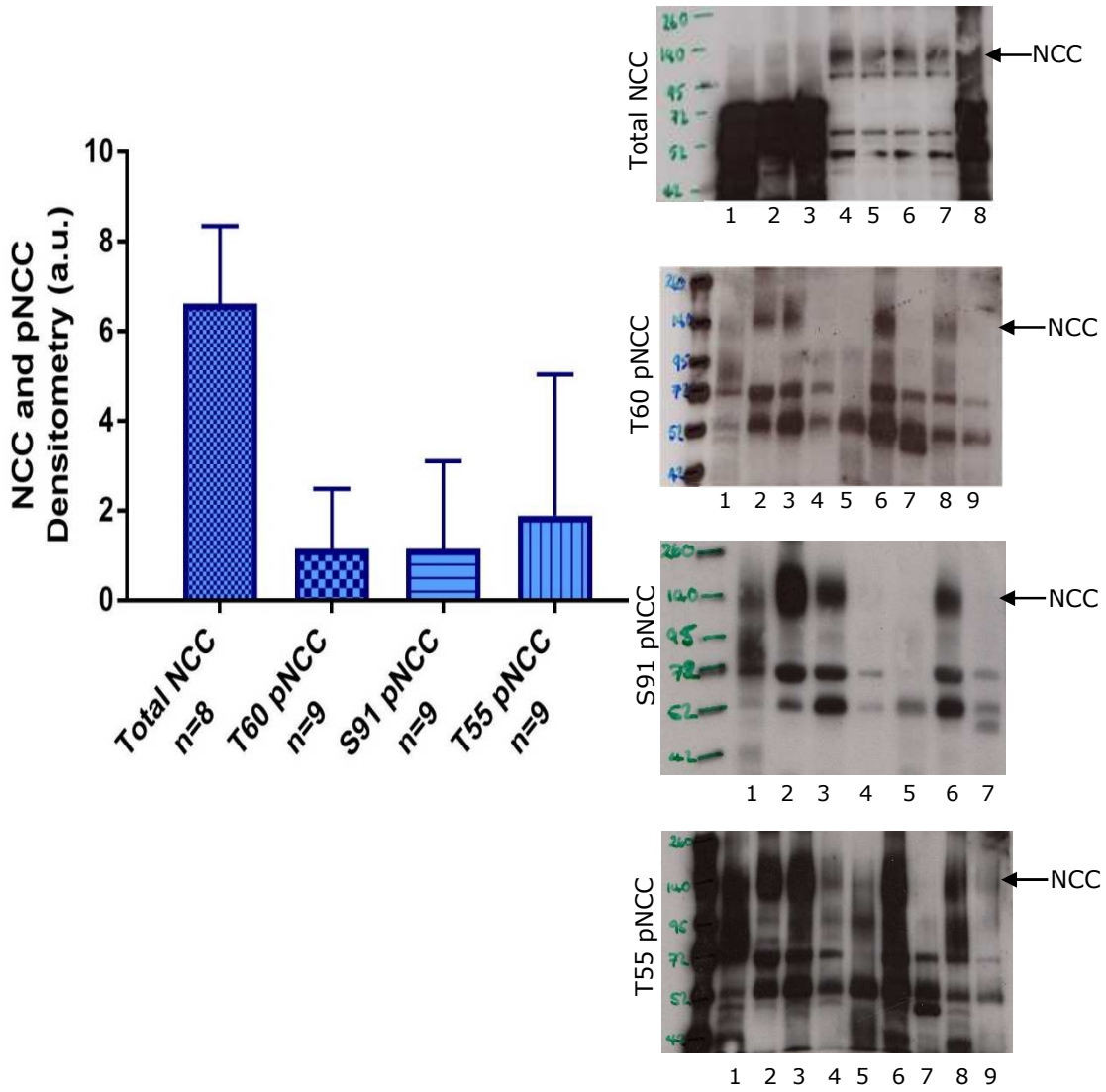


Figure 3-4 Comparison of expression of total NCC and pNCC in urine exosomes. *NCC and pNCC (S91, T60, and T55) are all expressed in the exosome fraction. Protein expression is the highest in Total NCC samples, with T55 pNCC expressing the highest of the phosphorylated proteins. No statistical analysis was performed.*

3.2.4 Positive and Negative controls

Expression of total NCC was compared in samples obtained from subjects with both Gordon and Gitelman syndromes, as shown in Figure 3-5. Patients with Gitelman syndrome express significantly less NCC than patients with Gordon Syndrome (0.8a.u.±0.7). Because of the large difference in expression, Gitelman syndrome samples will be used as negative controls, whereas samples from Gordon syndrome patients will be used as positive controls for further western blots. The blots that are shown below were produced by Dr Sarath Channavajjhala.

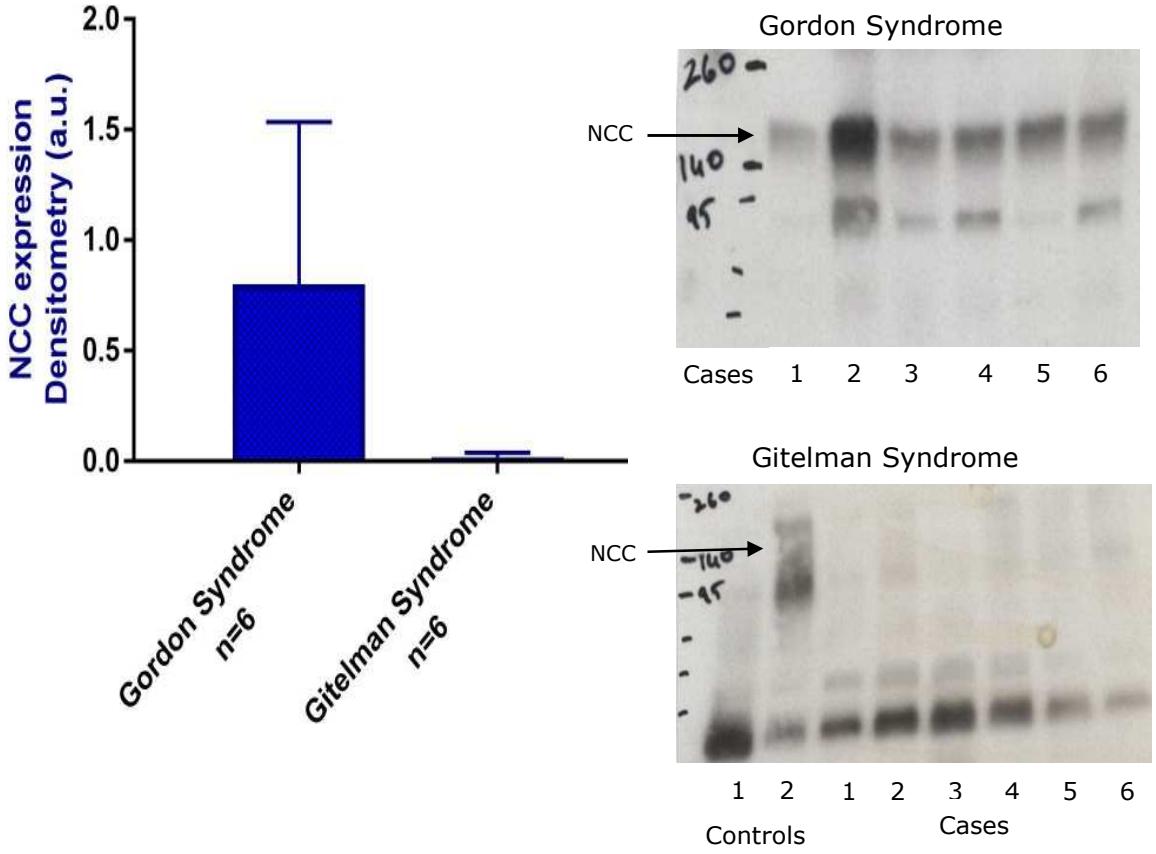
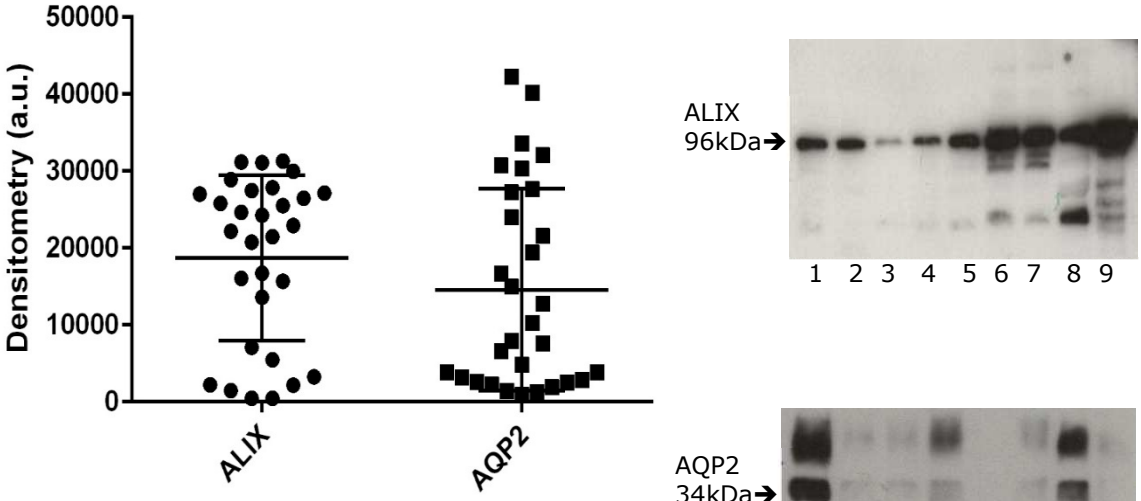


Figure 3-5 Comparing NCC expression between Gitelman and Gordon syndrome samples. *Gordon syndrome samples express significantly higher NCC protein than Gitelman Syndrome samples. Work was undertaken by Dr Sarath Channavajjhala and results shown here with permission.*

3.2.5 AQP2: an alternative to ALIX?

For the work described in future chapters, a house-keeper protein needed to be identified for urinary exosomes, so data produced could be normalised against a control. Within the field of exosomes, there is no control standard; therefore, a protein which has very little variation in expression and is found within urinary exosomes must be used. Both AQP2 and ALIX were considered for this investigation and were compared to determine which was most suitable.

ALIX proved to be more highly expressed than AQP2 by 20%, as well as having a higher average (18696.23a.u.±10772.25) as shown in Figure 3-6. As AQP2 may also vary in its expression in electrolyte and volume disorders, ALIX was the better control and was used to normalise against the following proteins.



ALIX		AQP2
18696.23	Average	14575.47
10772.25	StDev	13133.30
76.67	% of expressed	56.67

Figure 3-6 Comparison between ALIX and AQP2 expression. *The graph above shows that ALIX expresses in more samples than AQP2. The top blot shows protein expression of ALIX (~95kDa) from one of the gels. The bottom blot shows AQP2 (~34kDa) expression from one of the gels.*

3.3 The Role of Extracellular vesicles in Intercellular communications between HEK cells

3.3.1 HEK293 and related cell lines expression of NCC

The next section describes experiments designed to investigate possible extracellular communication between HEK 293 and primary epithelial Proximal Tubule Kidney Cells (PKTC).

Due to the growing evidence of exosome involvement in cell-to-cell communication, attempts were made to show the exosomal passing of NCC between HEK cell lines and PKTC. In total, 4 cell lines were used for this experiment:

- HEK-293 (Wild-Type)
- HEK-NCC (a transfected cell line which overexpresses NCC)
- HEK-EV (transfected empty-vector control for HEK-NCC)
- PKTC (to measure the biological effect)

Exosomes were harvested from the HEK cell lines and used to stimulate PKTC. Stimulation of PKTC with HEK exosomes was repeated three times. This chapter aims to show exosomes can alter the protein expression in a recipient cell by measuring changes in NCC expression in PKTC.

Attempts to generate the HEK-EV cell line were made, however, despite trying several exposure times, DNA and transfection chemical concentrations, and different degrees of cell confluency, producing a stable HEK-EV cell line failed. Communications with Prof D. Alessi's group, University of Dundee who generated the HEK-NCC cell were made, who kindly shipped a HEK-EV vial to our group. This cell line was used in this study.

3.3.1.1 NCC expression from HEK cell lines in 6-well plates

Validation experiments were performed to observe any exosomal expression of NCC from cells seeded in 6-well plates. At the time of seeding, 300k cells were plated into each well. Media supernatant was collected and collated per 6-well plate after 24 and 48 hours and frozen at -80°C.

As Figure 3-8 shows, no bands appeared in the exosome fractions. It was concluded that cells seeded at 300k per well did not produce enough exosomes, so the expression would not have been high enough to allow us to study PKTC cell NCC expression.

The blots also confirmed the heavily increased NCC/FLAG-NCC expression within transfected HEK NCC cell line compared to its wild type (HEK) and negative control (HEK-EV) cell lines (Figure 3-8).

3.3.1.2 NCC expression from HEK cell lines in T75 flasks

Given the low expression of exosomal NCC with cells seeded in 6-well plates, it was decided to repeat the experiment in T75 flasks [144]. This allowed for a higher number of cells, therefore the hope is an increased number of exosomes. Cells were counted after trypsinising.

Table 3-1 Cell counts of the 3 HEK cell lines in 4 T75 flasks. *Flask 1 will be used to measure NCC and FLAG-NCC protein expression. The exosomal supernatant of Flasks 2-4 will be used to stimulate PKTC cells.*

	Number of cells in each flask		
Cell line	**HEK WT**	**HEK-NCC**	**HEK-EV**
Flask 1	$4.50x10^6$	$3.58x10^6$	$8.73x10^6$
Flask 2	$7.58x10^6$	$4.08x10^6$	$3.40x10^6$
Flask 3	$4.78x10^6$	$3.61x10^6$	$2.1x10^6$
Flask 4	$4.18x10^6$	$3.53x10^6$	$4.23x10^6$

As levels of ALIX and AQP2 expression were too low to be quantified in exosomes from cells grown in 6-well plates and T75 flasks, β-actin was used as the house-keeper protein. β-actin is usually used as a house-keeping protein for cellular work and therefore deemed the most appropriate replacement for ALIX/AQP2. Densitometry values of β-actin were used to normalise NCC and FLAG-NCC expression.

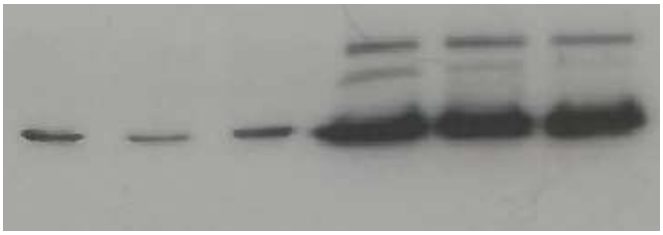


Figure 3-7 B-actin expression in HEK, HEK-NCC and HEK-EV exosomes and cell pellets. *Exosomal supernatant and cells were harvested from T75 flasks.*

Figure 3-9 shows that an increased cell number increases exosomal NCC expression and so can be quantified. HEK-WT and HEK-EV cell lines express a small amount of NCC, which is as expected. HEK NCC was therefore chosen as a positive control for NCC expression in PKTC cells.

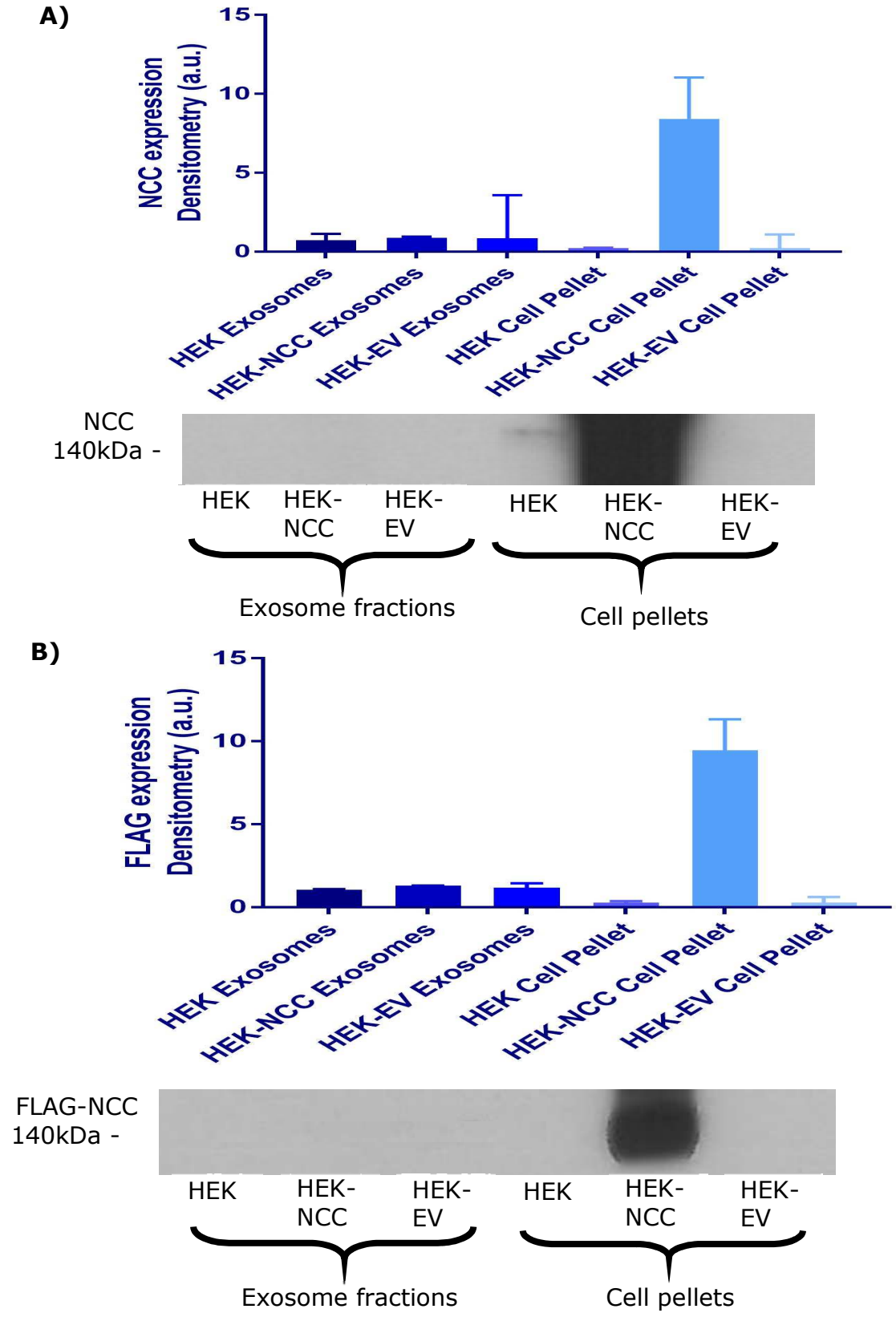


Figure 3-8 NCC and FLAG-NCC expression in HEK exosomes and cell pellets in 6-well plates*. A) NCC expression in HEK exosomes (normalised by exosomal β-actin) and cell pellet (normalised by cell pellet β-actin) from cells seeded in 6-well plates. B) FLAG-NCC expression in HEK exosomes (normalised by exosomal β-actin) and cell pellet (normalised by cell pellet β-actin) from cells seeded in 6-well plates.*

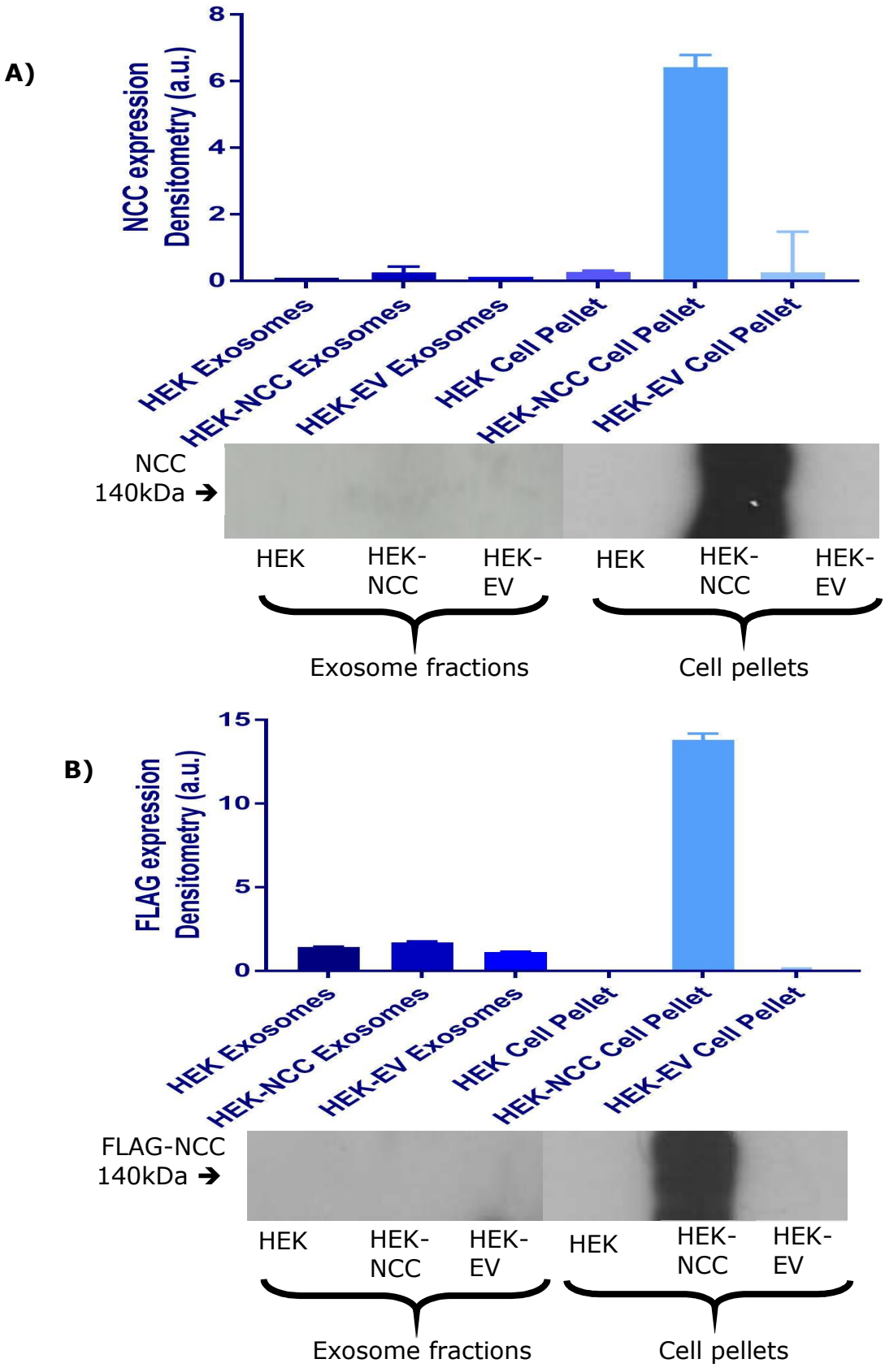


Figure 3-9 NCC and FLAG-NCC expression in HEK exosomes and cell pellets grown in T75 flasks. *A) NCC expression in HEK exosomes (normalised by exosomal β-actin) and cell pellet (normalised by cell pellet β-actin). B) FLAG-NCC expression in HEK exosomes (normalised by exosomal β-actin) and cell pellet (normalised by exosomal β-actin).*

3.3.2 NCC exosomal communication between HEK293 and Proximal Tubule Kidney Cells

Primary epithelial Proximal Tubule Kidney cells (PKTC) (ATCC; PCS-400-010) were chosen to measure the potential biological effect of exosomal communication, as they do not express NCC. Here 15mL of exosomal supernatant extracted from HEK cell lines were co-incubated with PKTC cell lines (Figure 3-9) to observe if a change of NCC expression was detected. Cells were stimulated at 70-80% confluency and incubated for 24 hours before cells and media were harvested.

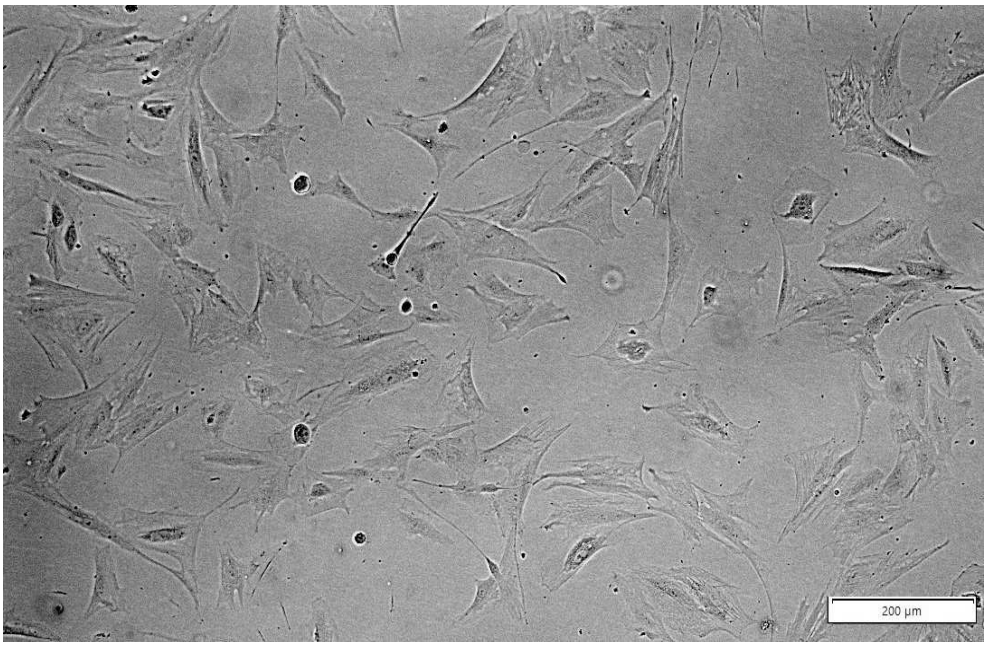


Figure 3-10 PKTC cells at 60-70% confluency under brightfield. *The picture was taken using an Olympus Inverted Microscope CKX53 at 10x magnification.*

To observe possible exosome uptake into PKTC, cells and exosomes were labelled with Qtracker® 655nm Cell Labelling Kit (red) and Qtracker® 565nm Cell Labelling Kit (green). Qdot nanocrystals diffuse through the cell membrane to fluorescently label the cells and exosomes without disrupting cell functions [145]. After 24 hours, cells were gently washed in PBS, harvested, and lysed. NCC and FLAG-NCC expression were measured using western blotting. Figure 3-10 shows PKTC cells (labelled green) accepted HEK exosomes after 24 hours of incubation. The presentation of red labelled exosomes within the PKTC cell membrane indicates that the contents (proteins as well as red nano-crystals) of HEK exosomes have entered into the PKTC cytoplasm.

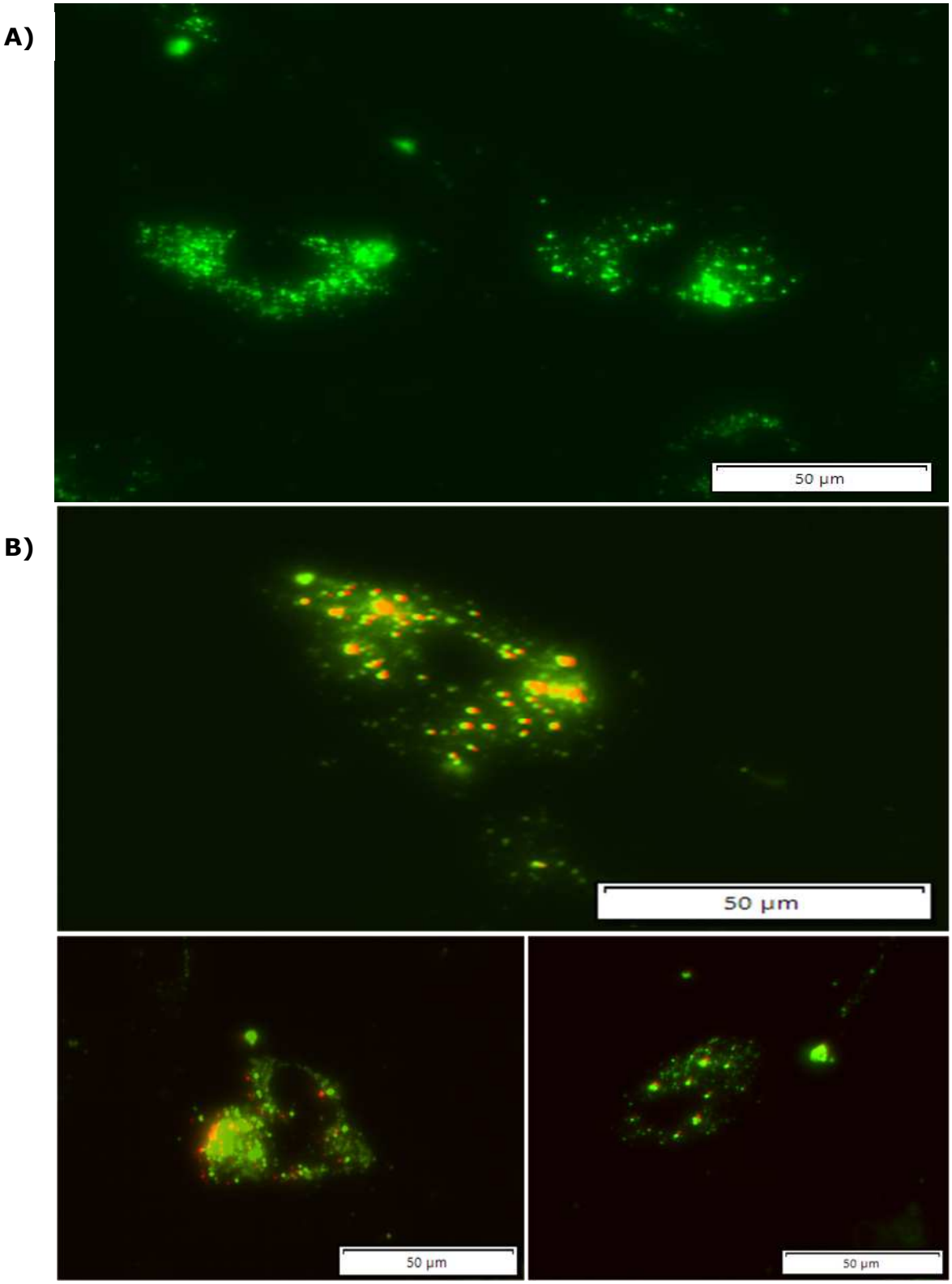


Figure 3-11 PKTC cells labelled with Qtracker® labelling kit. *A) PKTC cells before exosomal stimulation, labelled with Qtracker® 565nm (green). Pictures were taken on the Olympus Inverted Microscope CKX53. Magnification=40x. B) PKTC cells 24 hours after exosomal stimulation, exosomes labelled with Qtracker 655nm (red). Pictures were taken on the Olympus Inverted Microscope CKX53. Magnification=40x.*

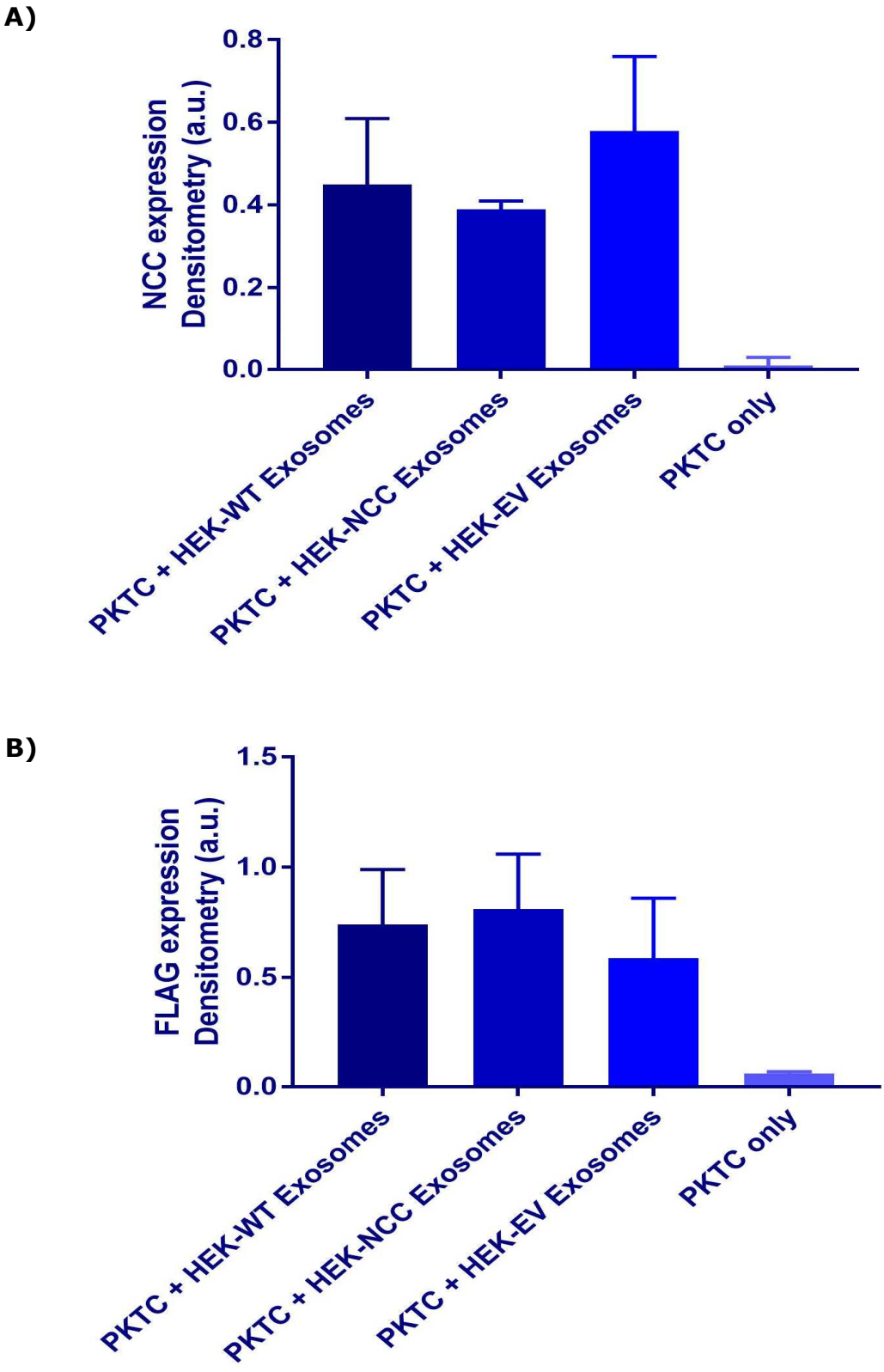


Figure 3-12 Expression of NCC and FLAG-NCC in PKTC cells with and without HEK exosomes (n=3). *A) NCC expression in PKTC cells; 3 cell lines with HEK exosome stimulation, 1 cell line with no stimulation. B) FLAG-NCC expression in PKTC cell lines; 3 cell lines with HEK exosome stimulation, 1 cell line with no stimulation.*

As expected, PKTC do not express any NCC (0.01a.u.±0.02) as shown by Figure 3-12. In PKTC cells that had been stimulated by HEK exosomes, increased NCC and FLAG-NCC expression was observed. This is in agreement with Figure 3-11 suggesting that the exosomes had transferred to PKTC cells. It was expected that only PKTC stimulated with HEK-NCC exosomes would express NCC or FLAG-NCC, however the results of Figure 3-12 suggest PKTC also expresses NCC with HEK-WT and HEK-EV exosomes. Therefore, PKTC does not show NCC-exosome dose-dependent expression of NCC. Although steps were taken to ensure all extracellular vesicles were removed, further experiments using a confocal microscope will be required to confirm if the HEK exosomes entered intracellularly.

3.4 Discussion

3.4.1 Optimal urine fractionation to study human exosomes NCC and AQP2

Urine has long been used as a source for protein analysis to assess glomerular filtration [1], however concentrating exosomes from urine allows for specific protein analysis to study renal pathophysiology in much more detail.

To optimise the best fraction created from the exosome extraction process, AQP2 and NCC were detected using western blots in urine samples from individuals with normal physiology (healthy volunteers). NCC and AQP2 expression were found almost exclusively in the exosomal fraction, with statistically differential expression ($p<0.05$) compared to the cell pellet and flow-through fractions.

Figure 3-2 confirms that the exosomal fraction is the optimal urine sample to study exosomal NCC and AQP2 protein and minimal expression of NCC and AQP2 was seen in the cell pellet and flow through. Other groups have confirmed the presence of NCC and AQP2 in the concentrated exosome fractions regardless of the extraction method [1, 118, 146].

Finally, as expected no expression was observed in the Flow Through fraction. The lack of expression within the Flow Through indicates the extraction process was successful at concentrating the exosomes in the vivaspin filter tubes.

3.4.2 Storage Conditions

Upon extraction of urinary exosome samples, NCC protein stability was maintained by storing at -80ºC. To optimise storage conditions, exosome samples from individuals with normal physiology were extracted from fresh urine or stored at -20°C or -80°C before extraction. These samples were compared using western blotting, to detect differences in NCC and AQP2 expression. Both -20°C and -80°C samples were extracted in the same conditions (temperature, time, location), but with the samples already having

a low expression level of NCC, the samples stored at -20°C suffered protein degradation, leaving little to no expression of both AQP2 and NCC.

NCC and AQP2 expressed in the fresh samples show no significant difference in abundance compared to exosome samples stored at -80°C. It can be concluded that storing urine samples at -80°C is preferable as it is considerably more convenient with the addition of no loss of protein expression when compared to extracting fresh urine samples.

3.4.3 Phosphorylated NCC

Figure 3.3 shows expression of the total NCC and NCC phosphorylated at specific phosphorylation sites (Proline60=P60, Serine91=S91 and Theronine55=T55), which are responsible for NCC activation. This result confirms the presence of total NCC and pNCC in the exosome fractions. As the four antibodies target different amino acid epitopes, there can be no meaningful comparison between the absolute abundance of each.

Despite epithelial cells in the DCT producing plenty of total NCC, not all are phosphorylated. This may explain why there appears to be a lower expression of T60, S91, and T55, as only a selected number of NCC are phosphorylated and activated at the apical membrane.

Most literature present data on total NCC, rather than phosphorylated NCC. The antibodies used in the data presented here target amino acids, which were first described in murine studies [146]. There are three principal N-terminal phosphorylation sites responsible for the regulation of NCC activity: T53, T58 and S71 [147]. The importance of these amino acids was highlighted when they substituted for Alanine in murine studies. This substitution causes the deactivation of NCC [147].

3.4.4 Positive and Negative Controls

Mutations found in Gordon Syndrome cause an increased expression of NCC at the apical membrane, whilst Gitelman syndrome mutations cause a loss-of-function and loss of expression of NCC. Comparing the expression of NCC between Gordon and Gitelman syndrome showed a clear expression difference, which demonstrates their validity as potentially positive and

negative controls, respectively. As an alternative to Gitelman syndrome samples, cell pellet or flow through samples can also be used as a negative control. As Gitelman syndrome samples can be quite precious, alternative samples are useful as long as NCC expression remains at very low concentration. This study has shown that these alternative samples produced throughout the extraction process can be used as negative controls.

3.4.5 Comparisons of exosomal markers

A recent study has suggested that ALIX is a suitable exosomal marker (or house-keeper protein) to standardise urinary exosome results [116]. Wolley *et*, al. have shown also shown the use of ALIX as an exosomal marker to standardise the analysis of exosomal transporter proteins e.g. NCC and NKCC2 in human urine [116]. With literature reports and the results from the study, ALIX may replace AQP2 as an exosomal marker in future proteomics work.

ALIX is a useful exosomal marker due to its role in creating exosomes. However, for ALIX to be used as an exosomal marker there must be a high protein concentration (as seen in Section 3.2). This was not the case for exosomes extracted from cells in culture, resulting in low protein concentration. Assuming there is enough protein present to measure, ALIX will be used to normalise protein expression in urinary exosomes. However, due to its high abundance, β-actin was used in this chapter due to the concentration of protein in cell culture. β-actin is a standard house-keeper protein in cell culture, hence the reason for using this protein after discovering the absence of ALIX and AQP2.

3.4.6 Exosomal communication between epithelial cells

The aim of the next series of experiments was to add to the growing evidence that exosomes play a cell-cell communication role by looking for the potential transfer of NCC. This experiment has shown through qualitative and quantitative data that NCC containing exosomes from a donor cell line are associated with expression of NCC by a recipient cell line.

The data presented in this chapter confirm previous observations that PKTC cells do not express NCC [148, 149]. Therefore, these primary cells were suitable for measuring if a non-endogenously expressed protein can be transferred via unrelated exosomes (i.e. as shown here from HEK cells). As the results showed, PKTC cells accepted exosomes without any external stimulation e.g. ionophore either via direct drug delivery or liposomal-drug delivery [150, 151], suggesting that the mechanisms by which exosomes entered the PKTC cells included spontaneous endocytosis or receptor-mediated endocytosis [152]. It would be interesting to undertake further experiments to see if NCC is functional following exosomal transfer.

Initial experiments aimed to extract exosomes from cells grown in 6-well plates, but with insufficient exosomal NCC, a new strategy was required. One previous study extracting exosomes from cell cultures used T225 flasks [144]. Therefore, we investigated the use of T75 flasks, as T225 flasks were not available. However, this change allowed more cells to be cultured leading to more exosomes being produced.

Unexpectedly, there was no difference in expression in NCC or FLAG-NCC between the 3 PKTC cell populations stimulated by the 3 different NCC-containing HEK exosomes (HEK, HEK-NCC and HEK-EV). One suggestion could be that PKTC expression of NCC is not dose-dependent, as may have been expected. However, it is also likely that there may have been NCC contamination in the HEK-WT and HEK-EV cells. The exosomal NCC results from Figure 3-8 and 3-9 show HEK-NCC to express the expected high-levels of NCC, whilst HEK-WT and HEK-EV express very low levels. However, this was not reflected in the recipient cells as seen in Figure 3-12. An experimental error may be the cause for this result e.g. contamination of exosomes or insufficient washing of exosomal incubated PKTC cells, and therefore the experiment should be repeated to confirm this.

The evidence presented in this chapter shows that exosomal content can be used for cell-cell communication, by transferring proteins (and potentially mRNA) to cells that do not endogenously express them. The result of this could change the genetic and epigenetic signalling of the donor cell [153]. This type of communication has already been researched in tumours, where

cancerous cells release exosomes, which are accepted by healthy cells. They have also been shown to be associated with metastasis [153].

There are some potential weaknesses in the data presented in this chapter. It would have been informative to quantify the number of exosomes stimulating the donor cell line, which could be carried out by flow cytometry or Nanosight. Experiments should also be repeated using confocal microscopy to confirm the exosomal uptake into the recipient cells.

Experiments could be repeated, but for longer, to characterise the timeline of exosomal stimulated recipient NCC expression. Other proteins that regulate NCC could also be tested in the recipient cell line (WNK1/4 and CUL3/KLHL3). Finally, functional experiments would be important to perform in the future to see whether or not exosome transfer of protein results in functional changes in the recipient cell. Cellular radiology experiments would be ideal for measuring the functionality of NCC, through the use of radioactive chloride. Experiments would be designed to measure the movement of radioactive chloride inside and outside of the cell to test if exosomal NCC was physiologically viable.

Chapter 4: Serum and Dietary potassium effects on expression of thiazide-sensitive sodium-chloride cotransporter within human urinary exosomes

4 Serum and Dietary potassium effects on expression of thiazide-sensitive sodium-chloride cotransporter within human urinary exosomes

Abstract

It has long been reported that a diet high in potassium can lower blood pressure. However, the molecular mechanisms to how potassium affects blood pressure is yet to understood. One hypothesis may lie with NCC, as the levels of expression change with high and low concentrations of potassium. Murine and human studies have shown that with increased serum potassium, NCC expression decreases. This chapter examines urinary exosome expression of NCC and its related proteins with the relationship with serum and dietary electrolytes in 30 normonatraemic patients with well-controlled hypertension.

Urinary exosomes were extracted from the 30 normonatraemic patients, where NCC and related proteins were quantified using western blotting and Image J. Characteristics, electrolyte concentrations and prescribed drugs were recorded in all patients, where the population were considered as near equal spread in gender with patients considered elderly. All samples collected from were not taking non-thiazide medication at the time.

A negative correlation was presented between phosphorylated NCC (S91 and T60) when correlated with serum potassium. However, no correlation was seen at all when serum potassium was compared to NCC_3 or $NCC_{1/2}$. In addition to serum potassium, 24hr urinary potassium did not correlate with NCC or phosphorylated NCC. Other electrolytes e.g. chloride, were correlated with NCC. It was identified that serum chloride negatively correlated with NCC_3, but not with phosphorylated NCC or $NCC_{1/2}$. No correlations were observed with any other serum or dietary electrolytes.

The data reported in this chapter confirms correlations between pNCC and serum potassium in normonatraemic hypertensive patients, which have previously been reported in murine models. An explanation to why other predicted correlations were not observed may be due to a lack of extreme potassium intake as seen in murine models, but this is challenging for any human studies due to adverse effects.

4.1 Introduction

With hypertension becoming increasingly prevalent worldwide, there is an obvious demand for further understanding of the regulation of blood pressure. Healthy lifestyle advice for patients with hypertension includes the consumption of fruit and vegetables regularly which is thought to be beneficial in helping to lower blood pressure, due to their high potassium concentration [60]. The Dietary Approaches to Stop Hypertension (DASH) study shows that increased dietary fruit and vegetable intake results in lower blood pressure [60].

The expression of NCC within the DCT has been shown to reduce with dietary potassium supplementation [54]. Murine studies have also shown that increased serum potassium concentration leads to a decreased expression of NCC, whilst decreased concentrations of serum potassium lead to increased expression of NCC [154, 155]. As mentioned in Section 1.11.2, increased dietary potassium intake has been shown to decrease blood pressure in rodent and human studies [53-58]. Although the specific mechanism is still unclear, a reduction in NCC expression may be partially responsible for the observed reduced blood pressure, as it would be expected to reduce reabsorption of NaCl from the DCT.

The expression of NCC is regulated via two mechanisms or modalities within the cells of the distal nephron. Type I regulation is responsible for NCC expression, whilst Type II regulates the activation/phosphorylation of NCC.

Type I regulation involves changes at the level of the transcription and translation of the protein, which is activated through the WNK-SPAK/OSR1-SPAK cascade. However, as with many transport proteins, NCC activity is minimal within the cell or cell membrane until phosphorylated. Type II regulation is mediated by the phosphorylation of key amino acids [109]. These include serine (S91) and threonine (T55 and T60) sites at the N-terminus of NCC [156]. The addition of phosphoryl groups activates NCC allowing Na^+ and Cl^- to pass through the cell membrane.

Although the WNK-SPAK-NCC cascade is at the centre of regulation of both pathways, Type I NCC and Type II NCC regulation are down-regulated in different ways. If NCC has not been trafficked to the cell membrane (Figure 4-1), WNK4 diverts NCC from the Trans-Golgi Network to be degraded by lysosomes [109]. However, active NCC (phosphorylated by SPAK via Type II regulation) is reabsorbed back into the cell through endocytosis [109]. At this point, NCC can be absorbed into exosomes as cargo, as described in Figure 1-6.

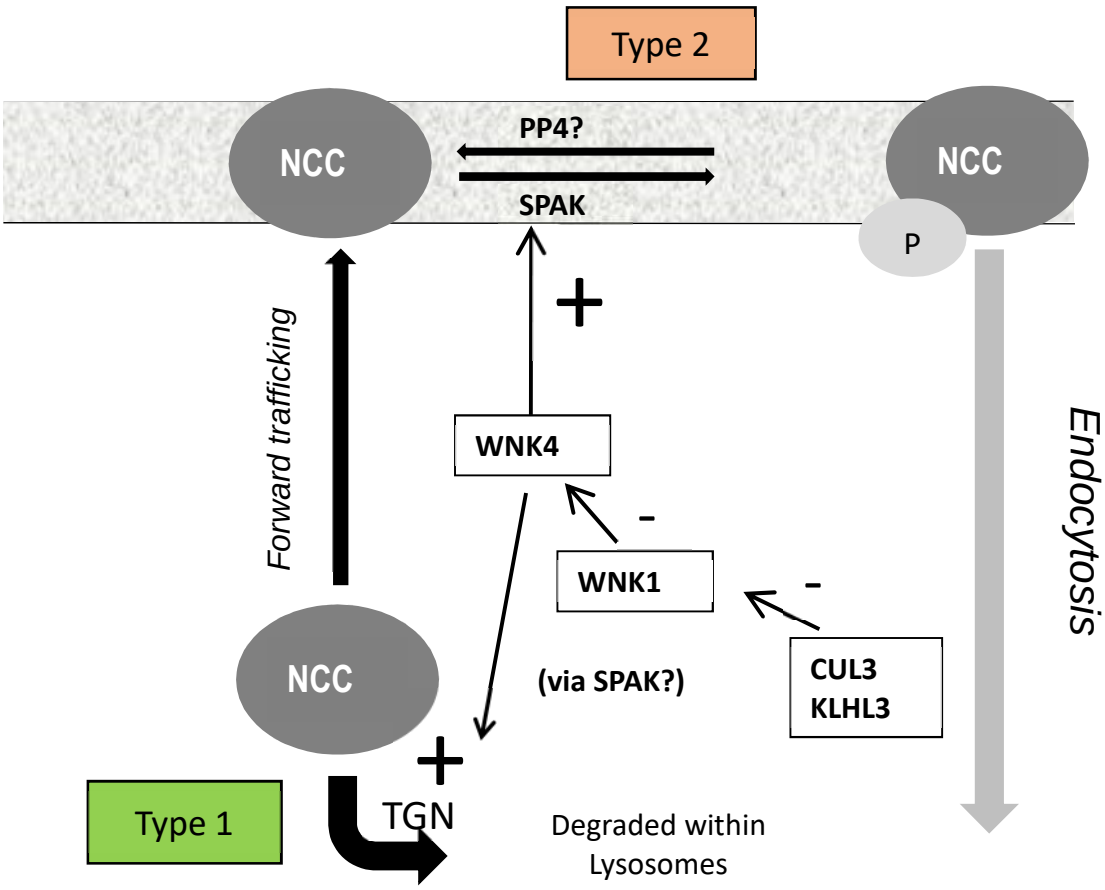


Figure 4-1 Mechanisms of regulating NCC. *The WNK-SPAK-NCC cascade is responsible for regulating Type 1 and Type 2 regulation of NCC. As well as phosphorylating SPAK, WNK also downregulates NCC by diverting the protein from the Trans-Golgi Network (TGN). This prevents further post-translation modifications and transportation to the cell membrane. Type II regulation involves the addition of a phosphoryl group to NCC by SPAK. The removal of phosphoryl groups may be caused by Protein Phosphatase 4 (PP4). The figure was adapted from Glover and Clayton, 2012 [109].*

The regulation of NCC and pNCC is dependent on Angiotensin II and aldosterone. These hormones phosphorylate SPAK, as well as WNK4, essentially converting WNK4 from inhibiting NCC to activating NCC [157, 158]. With increased activation of SPAK and WNK4, this leads to increased NCC

expression and activation [157, 158]. The expression and activation of the protein increases with increased concentrations of aldosterone in the blood, proven in individuals with hypertension secondary to hyperaldosteronism [54].

In this chapter, every patient's aldosterone and renin levels were measured. Patients with elevated aldosterone to renin ratio were not included in this study.

Ex vivo, rodent models have shown a high potassium diet results in dephosphorylation of NCC within the DCT cells [154]. To fully understand whether potassium and other electrolytes affect NCC expression, more studies are required to measure the human regulation of NCC expression. Using urinary exosomes from human patients, this chapter explores the relationship between the WNK-SPAK-NCC pathway and serum potassium concentration/urinary dietary electrolyte intake (as estimated from 24hr electrolyte excretion) [159].

In order to explore this relationship, this chapter examines urinary exosome expression of key proteins and the relationship with serum and dietary electrolytes in 30 normonatraemic patients with well-controlled hypertension.

4.2 Characteristics of normonatraemic hypertensive patients

A Power calculation was carried out to identify the number of subjects required for correlations presented in Chapter 4. As the primary analysis was to compute Pearson's correlation for the associate between 2 continuous variables, Nquery Advisor A was used to generate a sample size by inputting three variables:

- Power=80%
- Effect size=0.5 (50%) (50% increase or decrease in protein expression)
- Significance=0.05

Informed by the power calculation described above, 30 normonatraemic patients with well-controlled hypertension off thiazides were selected from the TIH study control group. Information about each patient was gathered upon the collection of the blood and urine samples. And the volunteers were split into even numbers by gender. All physiological parameters were within normal ranges, except systolic BP was mildly elevated in some patients, as shown in Table 4-1. Urine creatinine was also measured to ensure accuracy within the 24-hour urine samples.

Table 4-1 Descriptive data of characteristics of normonatraemic, non-thiazide volunteer patients. *Age, gender, blood pressure, pulse, weight, height, and BMI were measured at the time of donation of blood and urine. Kidney function and electrolyte concentrations were measured at the NUH Biochemistry Laboratory. Data is presented as averages and Standard Deviations.*

Characteristic	Value
Age	71±8.3yrs
Gender	Male 16
	Female 14
Systolic BP	145.1±18.7mmHg
Diastolic BP	79.4±9.2mmHg
Average Pulse	70.6±0.5 beats/min
Weight	78.3±17.6kg
Height	1.7±0.1m
BMI	27.2±4.6kgm
Kidney Function (eGFR)	76.36±11.6mL/min
Serum Sodium	138.7±1.8mM
Serum Potassium	4.16±0.3mM
Serum Chloride	100.1±18.8mM
Urine Creatinine	6.42±3.99mM
24hr Sodium	100.1±43.4mmol/24hrs
24hr Potassium	76.8±30.3mmol/24hrs
24hr Chloride	114.0±51.8mmol/24hrs
24hr urine volume	1783.1±518.4mL/24hrs

Table 4-1 shows that the study group consisted of a near equal spread in gender with patients generally being elderly. Overall, most patients had well-treated hypertension using non-thiazide medication; principally 'A' and 'C' type medications. All serum and urinary electrolytes were within the normal ranges.

As diuretics can affect the function of NCC and Na^+ reabsorption, the patient samples used in this study were not taking any form of diuretics.

Table 4-2 describes the anti-hypertensive drugs taken by the selected patients. As would be expected, patients were treated using ACE-inhibitors and calcium-channel blockers, and a quarter of patients were on combinational therapy.

Table 4-2 Percentages of anti-hypertensive drugs taken. *It was important to exclude any patients taking thiazide or loop diuretics due to their interference with NCC (NKCC1/2 inhibition can alter downstream NCC function). The drugs listed below are used in the first two steps of controlling blood pressure, as described in Figure 1-5. Dual combination refers to the combination of two drug types, of either Beta-blockers, ACE-inhibitors, calcium-channel blockers or angiotensin-II receptor antagonist.*

Drug Type	Percentage
Beta-blockers	20%
ACE-inhibitors	40%
Calcium-channel blockers	40%
Angiotensin-II receptor antagonist	23%
Dual combination	26%

4.3 Correlation between electrolyte, and exosomal protein expression

The concentration of serum and 24hr electrolyte data were correlated with the exosomal protein expression of NCC_3, $NCC_{1/2}$, phosphorylated NCC (pNCC S91, T60 and T55), WNK4, SPAK and ROMK.

Western blotting and Image J densitometries were performed to quantify urinary exosomal proteins NCC_3, $NCC_{1/2}$, pNCC (T60, T55, and S91), WNK4, ROMK, and SPAK as described in Section 2.4. The correlations between urinary electrolytes and proteins were calculated through Pearson's correlation and presented in a scatter plot, with the line of best fit to show the correlation. The electrolytes analysed were those previously described as having a potential role in the regulation of blood pressure: serum potassium, serum sodium, serum chloride, serum calcium, dietary sodium, dietary potassium and dietary chloride.

4.3.1 24hr urinary potassium excretion vs Serum potassium

It is assumed that the daily intake of dietary potassium is similar to 24-hour excretion via the kidneys at a steady state [159]. Despite the recent publication by Birukov et al. in this study, the 24hr urinary potassium excretion will be used as a surrogate potassium intake [160].

Because of the suggestion by previous mouse models that dietary potassium may determine or influence the concentration of serum K^+ [154, 161], correlations were explored between each individual's 24hr potassium excretion and serum K^+ in our human volunteers (Figure 4-2). In this analysis, no correlation was seen between 24hr potassium excretion and serum potassium (R^2=0.0004, p>0.05).

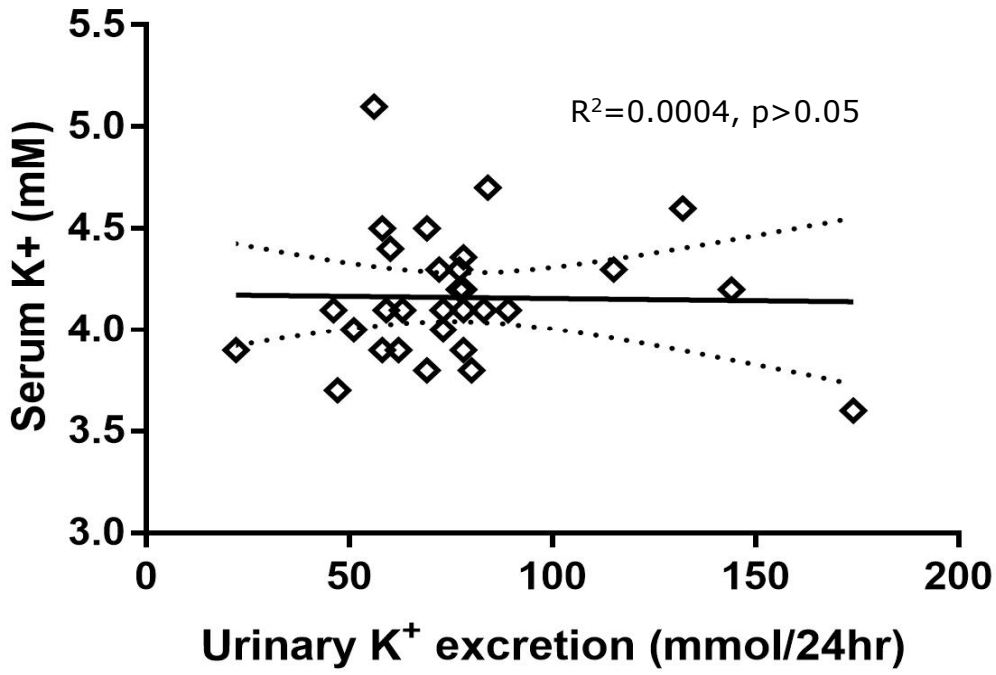


Figure 4-2 Correlation between 24hr K^+ excretion and Serum K^+ concentration. *Each point represents an individual patient. There was no correlation between 24hr potassium excretion and serum K^+ in this study.*

4.3.2 NCC_3

Previous literature shows potassium causes a decrease in the expression of NCC [116, 154]. Figure 4-3 shows that there was a trend towards a negative correlation between serum potassium concentration and NCC_3 expression; however, this was not significant. Figure 4-3 also shows a non-significant correlation between serum sodium and NCC_3.

Serum chloride and serum sodium are transported across the apical membrane of the DCT in the same direction, via NCC [162]. A negative correlation was seen which was statistically significant (R^2=0.200, p=0.0131).

Given literature suggesting that calcium and calcium-dependent proteins are involved in the regulation/dephosphorylation of NCC_3, investigations were carried out to see if serum calcium was correlated with NCC_3 expression. Figure 4-3 shows no significant correlation between serum calcium concentration and NCC_3.

It is assumed that the daily intake of dietary potassium is similar to 24hr excretion via the kidneys at a steady state [159]. No significant correlation was seen between either 24hr urinary potassium excretion, 24hr urinary sodium excretion or 24hr urinary chloride excretion and NCC_3 expression.

4.3.3 $NCC_{1/2}$

The majority of the literature describes electrolyte relationships with NCC_3; however, 45% of the total human NCC is isoform $NCC_{1/2}$ [20]. It would be expected that $NCC_{1/2}$ follow similar patterns of regulation to NCC_3. Surprisingly Figure 4-4 shows $NCC_{1/2}$ expression shows no correlation with any electrolyte, suggesting that only NCC_3 is affected by electrolytes.

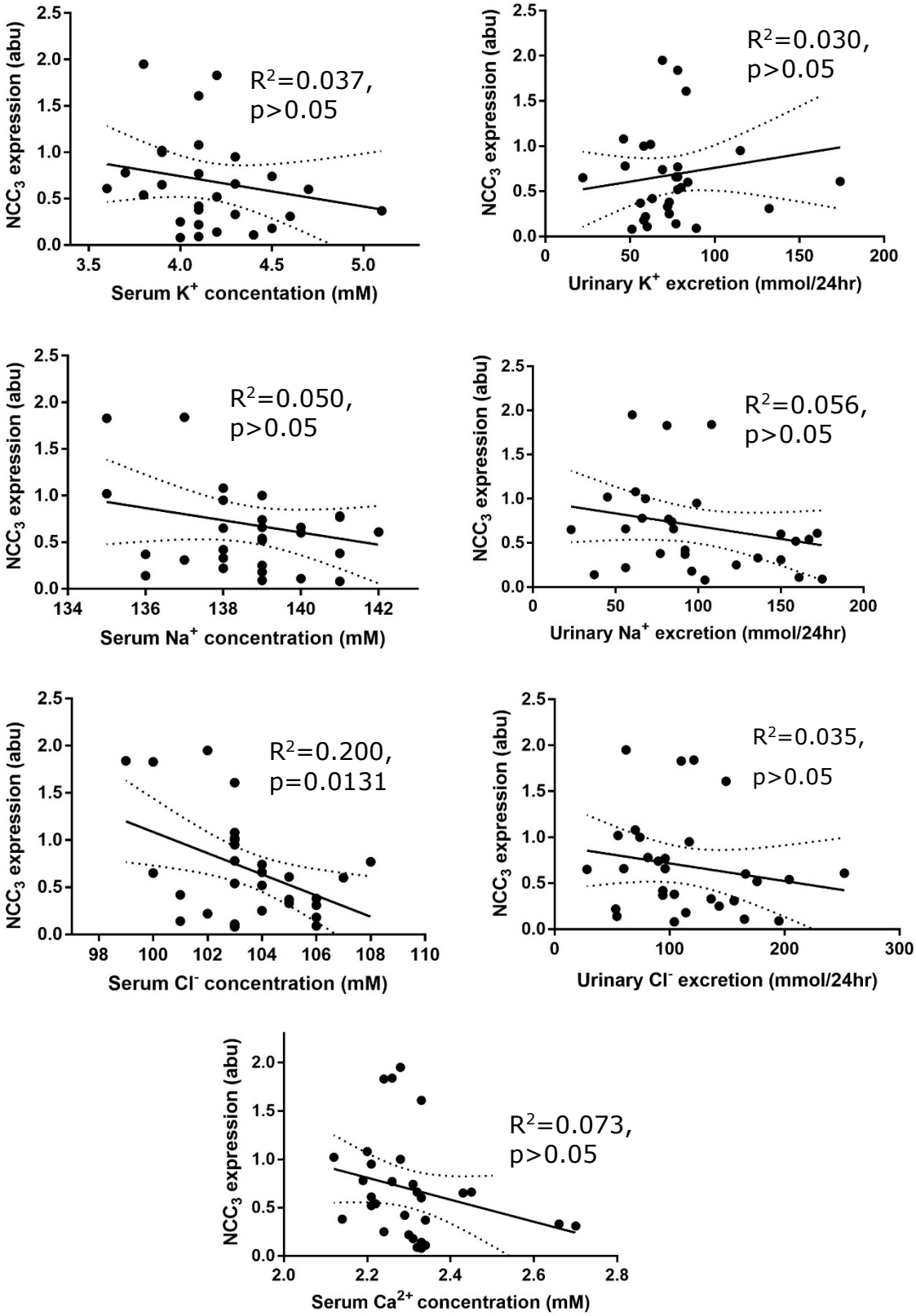


Figure 4-3 Linear correlations of NCC_3 between Serum K^+ concentration, Serum Na^+ concentration, Serum Cl^- concentration, Serum Ca^{2+} concentration, 24hr Na^+ excretion, 24hr K^+ excretion, and 24hr Cl^- excretion. *Each individual point represents a patient. Abu = Arbitrary units.*

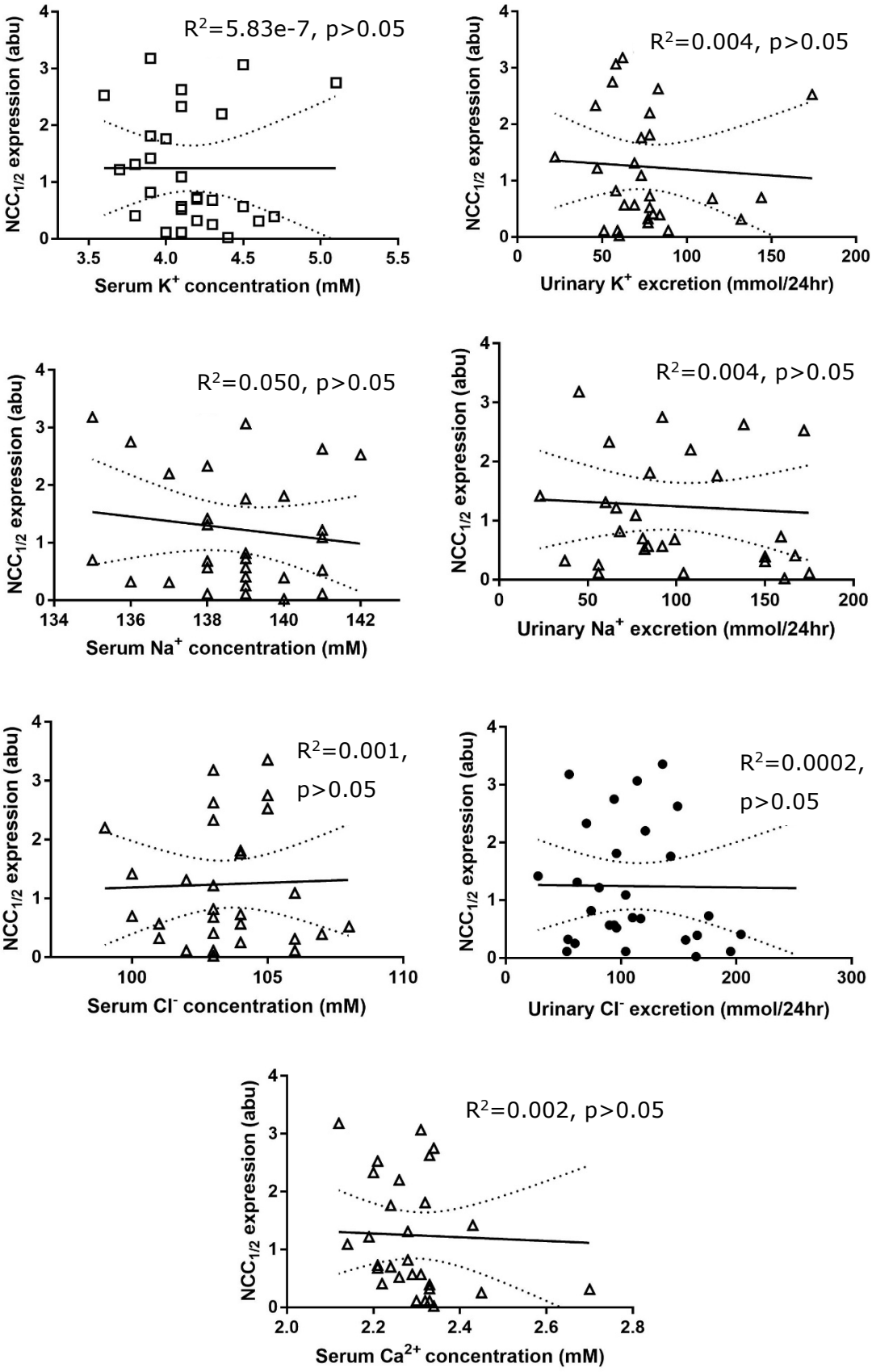


Figure 4-4 Linear correlation between $NCC_{1/2}$ and Serum K^+ concentration, Serum Na^+ concentration, Serum Cl^- concentration, Serum Ca^{2+} concentration, 24hr Na^+ excretion, 24hr K^+ excretion, and 24hr Cl^- excretion. *Each individual point represents a patient. Abu = Arbitrary units.*

4.3.4 pNCC

There is evidence in previous literature that serum potassium concentration modulates the expression of NCC and pNCC in mouse models [154]. Here the three phosphorylation sites (S91, T55 and T60) were measured separately in addition to total phosphorylated NCC reported in other studies. These 3 amino acids, which are responsible for NCC activation within the N-terminal [117], were quantified and their correlations were explored against specific electrolytes levels.

4.3.4.1 pNCC S91

As hypothesised, Figure 4-5 shows serum potassium concentrations inversely correlate with the expression of the active pNCC S91 protein (R^2=0.150, $p<0.05$), with statistical significance.

Although the correlation between serum calcium concentration and pNCC S91 expression was not significant, the p-value was just above the threshold of 0.05 ($p=0.0505$). It is possible that with a larger sample size, the observed negative correlation could be a representation of physiological changes (R^2=0.1298).

Unlike NCC_3, Figure 4-5 also shows the correlations between serum sodium and serum chloride concentration with pNCC S91 were non-significant. Correlations between 24-hour potassium excretion and pNCC S91 were also not significant, which was unexpected when considering previous reports [154, 155].

4.3.4.2 pNCC T55

Unlike pNCC S91, Figure 4-6 shows a non-significant trend against pNCC T55 and potassium concentration, as the correlation between the two parameters was not as strong. 24hr potassium excretion showed a strong and unexpectedly positive correlation with pNCC T55 (R^2=0.337, p=0.0008). This correlation shows with low urinary potassium excretion concentrations, there is also a low expression of pNCC T55, but with high urinary potassium excretion, there is a high level of pNCC T55 expression. The remaining electrolytes (serum sodium, serum calcium, serum chloride, and dietary sodium) correlated with T55 showed no correlation or significance.

4.3.4.3 pNCC T60

As Figure 4-7 shows, pNCC T60 has a negative correlation with serum potassium (R^2=0.166, p=0.0252). The data suggests that with low concentrations of serum potassium, there is a high expression of pNCC T60, whilst with high serum potassium, there is low pNCC T60 expression. Serum sodium, serum chloride, serum calcium, 24hr sodium excretion, 24hr potassium excretion and 24hr chloride excretion have no significant correlation with pNCC T60.

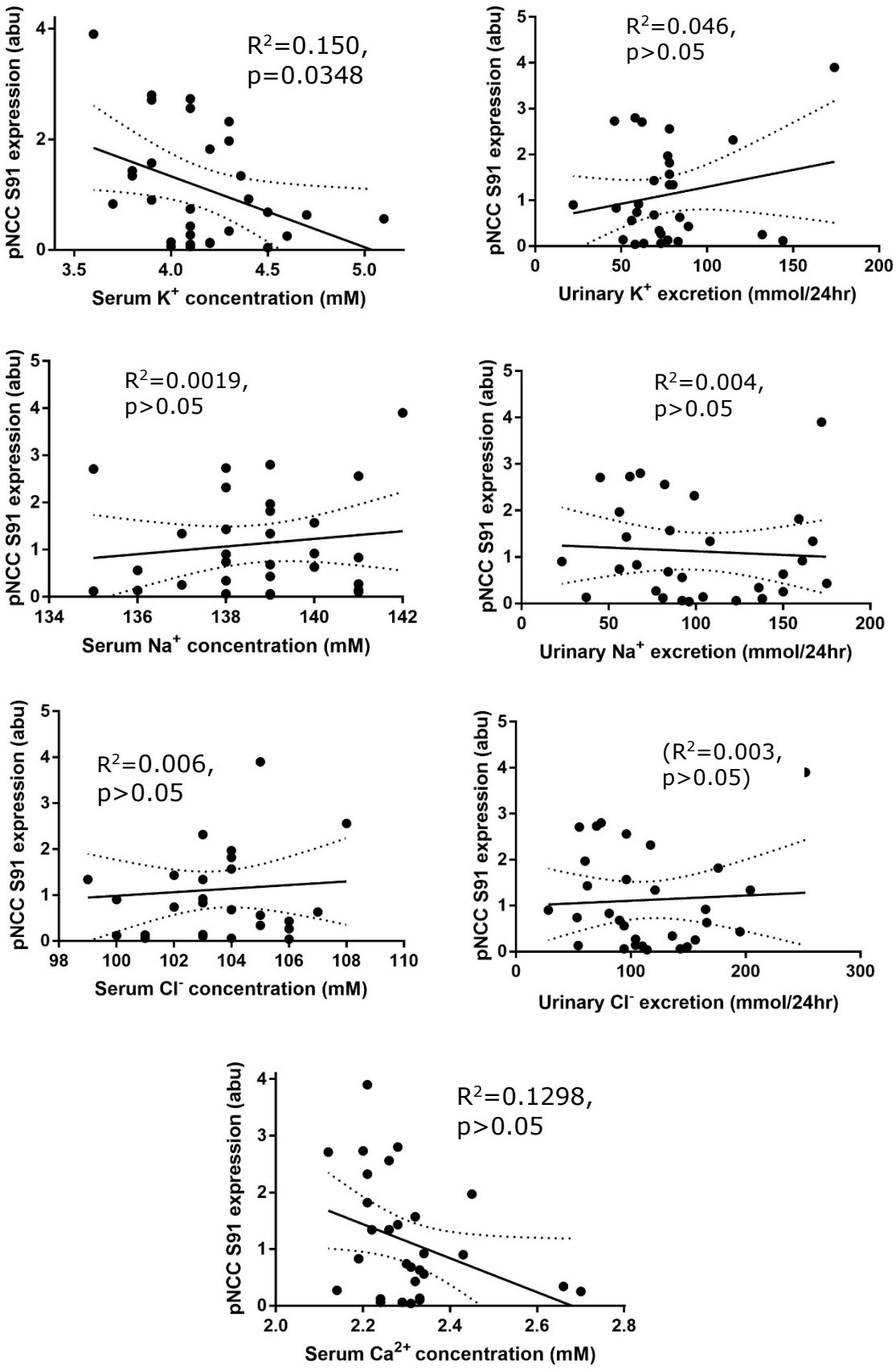


Figure 4-5 Linear correlation between pNCC S91 and Serum K^+ concentration, Serum Na^+ concentration, Serum Cl^- concentration, Serum $Ca2^+$ concentration, 24hr Na^+ excretion, 24hr K^+ excretion, and 24hr Cl^- excretion. *Each individual point represents a patient. Abu = Arbitrary units*.

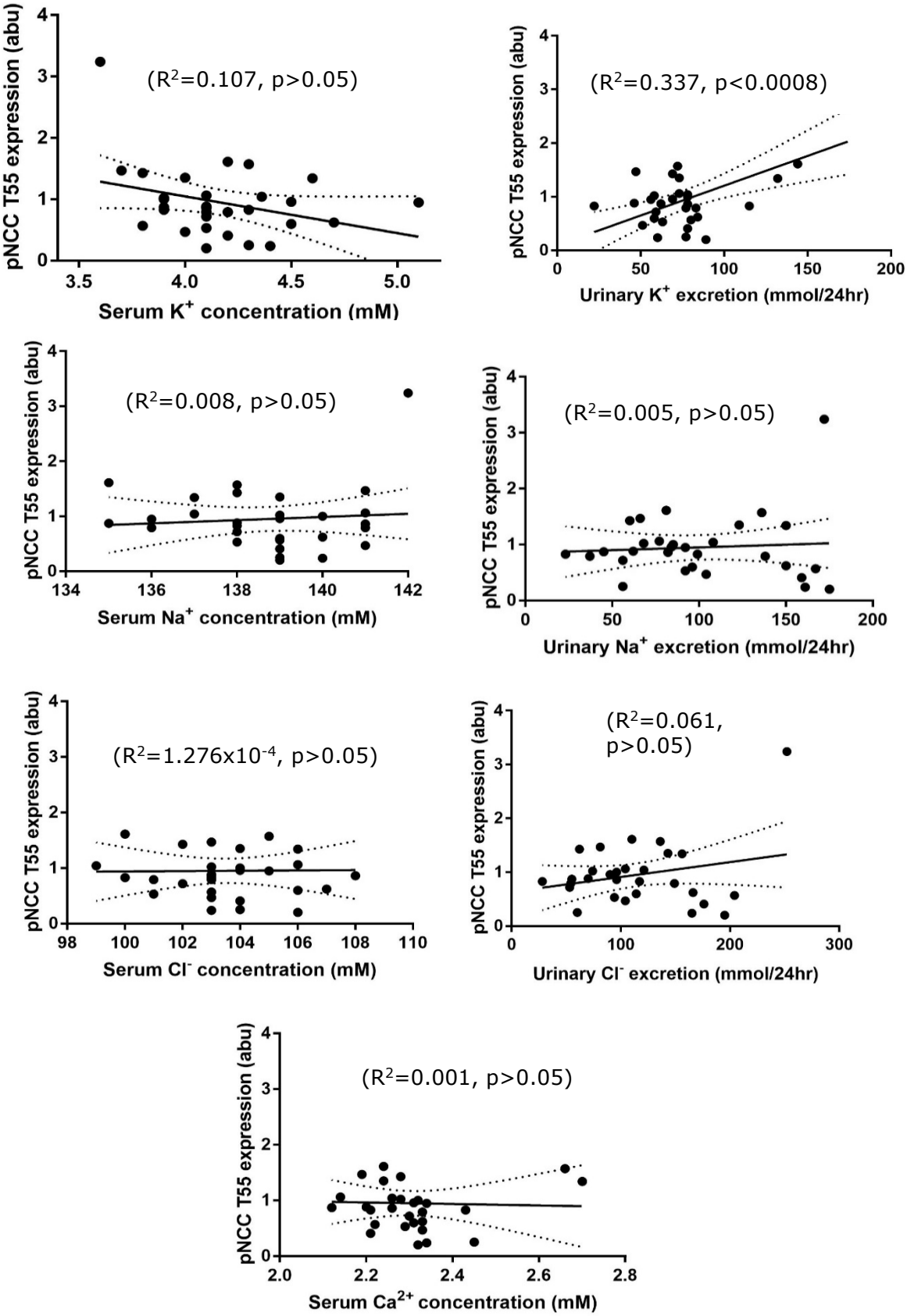


Figure 4-6 Linear correlation between pNCC T55 and Serum K^+ concentration, Serum Na^+ concentration, Serum Cl^- concentration, Serum $Ca2^+$ concentration, 24hr Na^+ excretion, 24hr K^+ excretion, and 24hr Cl^- excretion. *Each individual point represents a patient. Abu = Arbitrary units.*

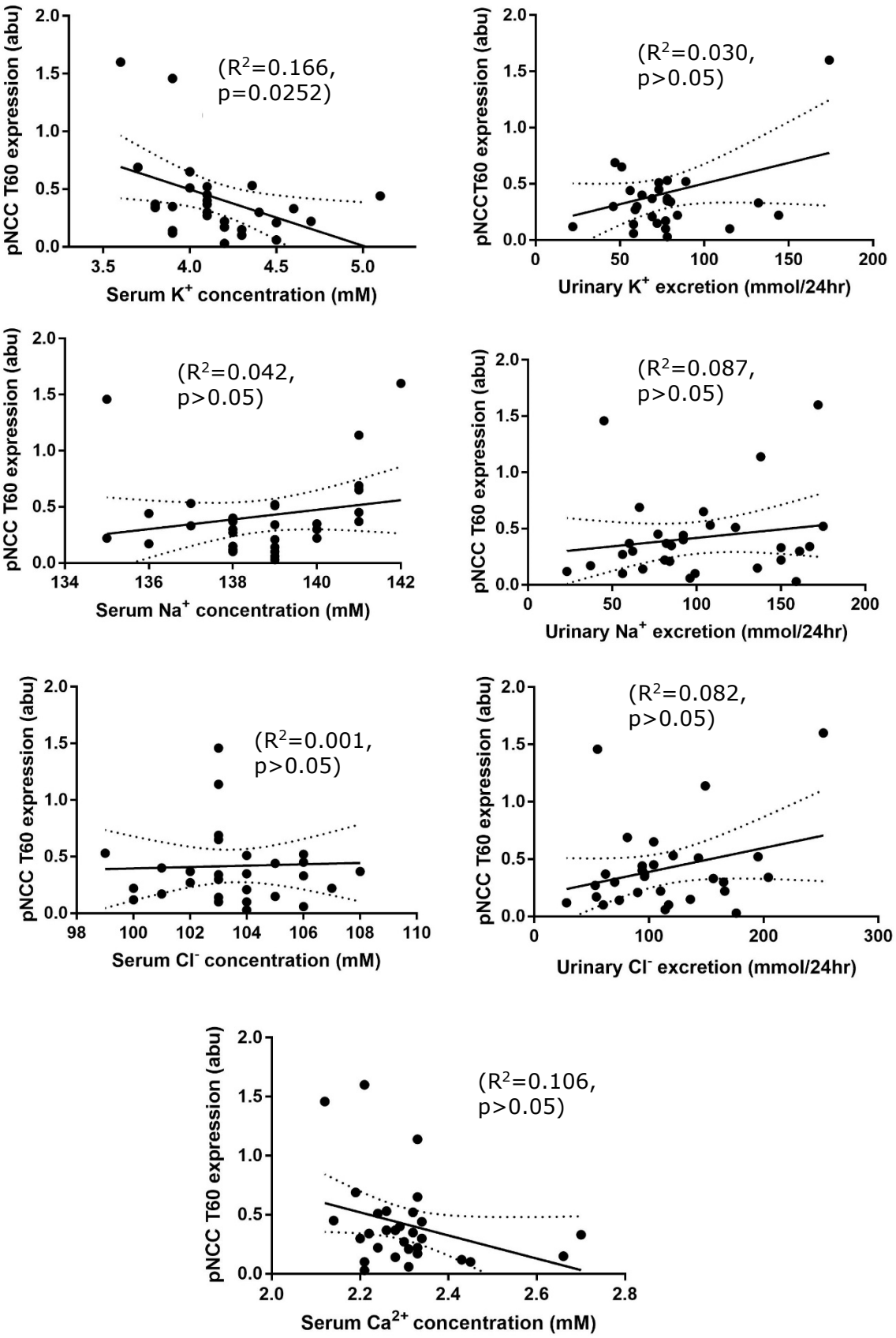


Figure 4-7 Linear correlation between pNCC T60 and Serum K^+ concentration, Serum Na^+ concentration, Serum Cl^- concentration, Serum Ca^{2+} concentration, 24hr Na^+ excretion, 24hr K^+ excretion, and 24hr Cl^- excretion. *Each individual point represents a patient. Abu = Arbitrary units.*

4.3.5 SPAK

As SPAK is involved in the regulation of NCC through the WNK-SPAK-NCC cascade, possible correlations were also explored between exosomal SPAK expression and electrolyte levels [163]. Figure 4-8 shows 24hr sodium excretion (R2=0.187, p=0.0171) and 24hr chloride excretion (R2=0.160, p=0.0285) have a significant negative correlation with SPAK.

From the graph presented in Figure 4-8, exosomal SPAK is more highly expressed with low 24hr sodium excretion and low 24hr chloride excretion concentrations, but there is a reduced expression of the protein when 24hr sodium excretion and 24hr chloride excretion concentrations are high.

The remaining associations made between SPAK and the other electrolytes were not significant, showing no correlations between the variables. However, the correlation between 24hr potassium excretion and SPAK produced a p-value of 0.0594. Due to the p-value lying so close to the 0.05 cut off, it is possible this may be a true negative correlation between the 24hr electrolyte and exosomal SPAK expression (R^2=0.121). However, without further studies with an increased sample size, this association cannot be confirmed.

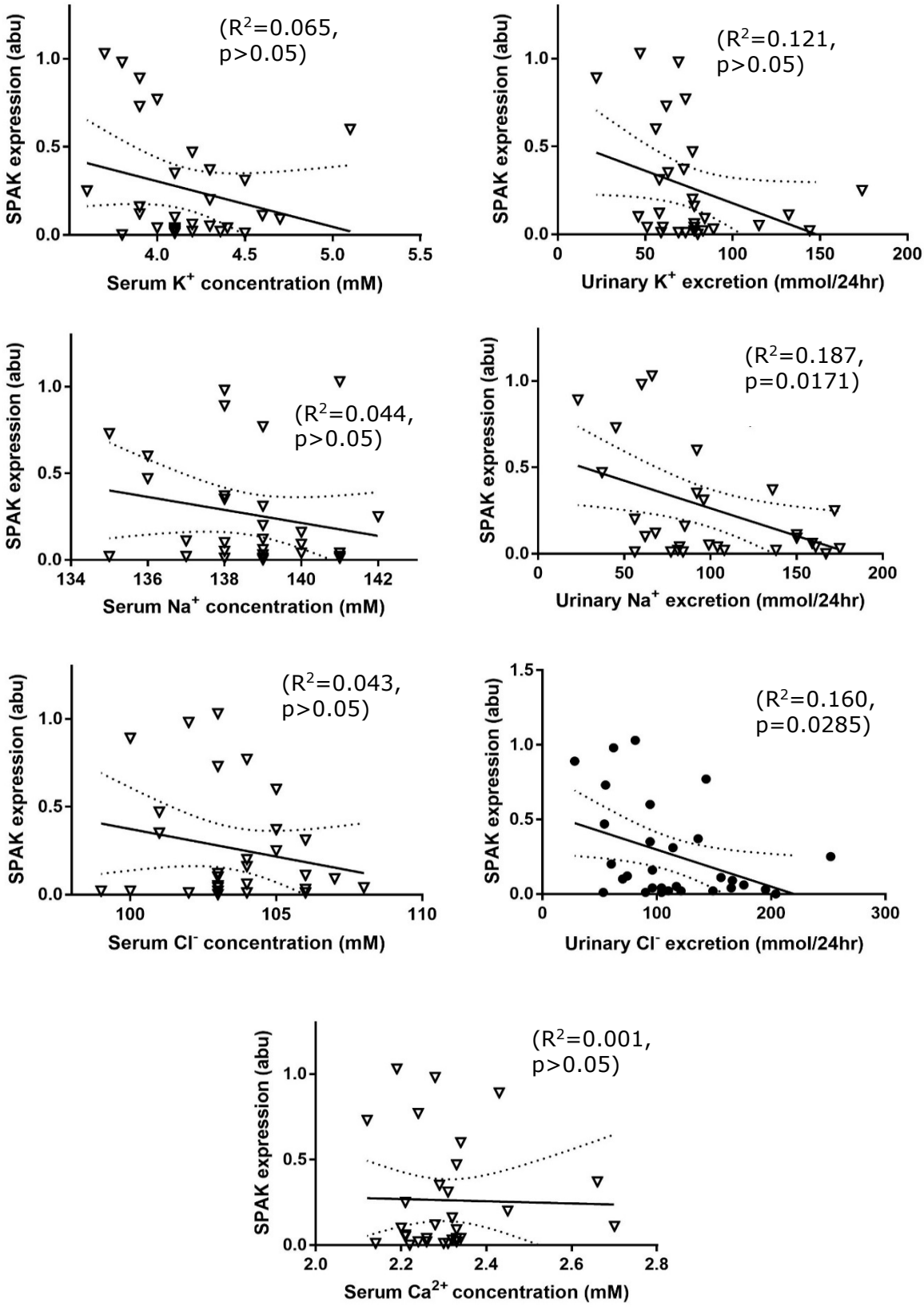


Figure 4-8 Linear correlation between SPAK and Serum K^+ concentration, Serum Na^+ concentration, Serum Cl^- concentration, Serum Ca^{2+} concentration, 24hr Na^+ excretion, 24hr K^+ excretion, and 24hr Cl^- excretion. *Each individual point represents a patient. Abu = Arbitrary units.*

4.3.6 ROMK

As ROMK is a potassium channel, which causes potassium reabsorption from the CD, it is possible that its expression could also be affected by serum and dietary potassium, as well as other electrolytes [164].

As Figure 4-9 shows, within normal electrolyte ranges, no significant correlations were observed between ROMK expression and any electrolyte, including serum potassium.

4.3.7 WNK4

To complete the full WNK-SPAK-NCC cascade, WNK4 expression was measured, with the aim of correlating it to the key electrolytes. However, no expression of WNK4 was observed in any sample. After repeating western blotting several times, it was concluded that WNK4 was not expressed in urinary exosomes.

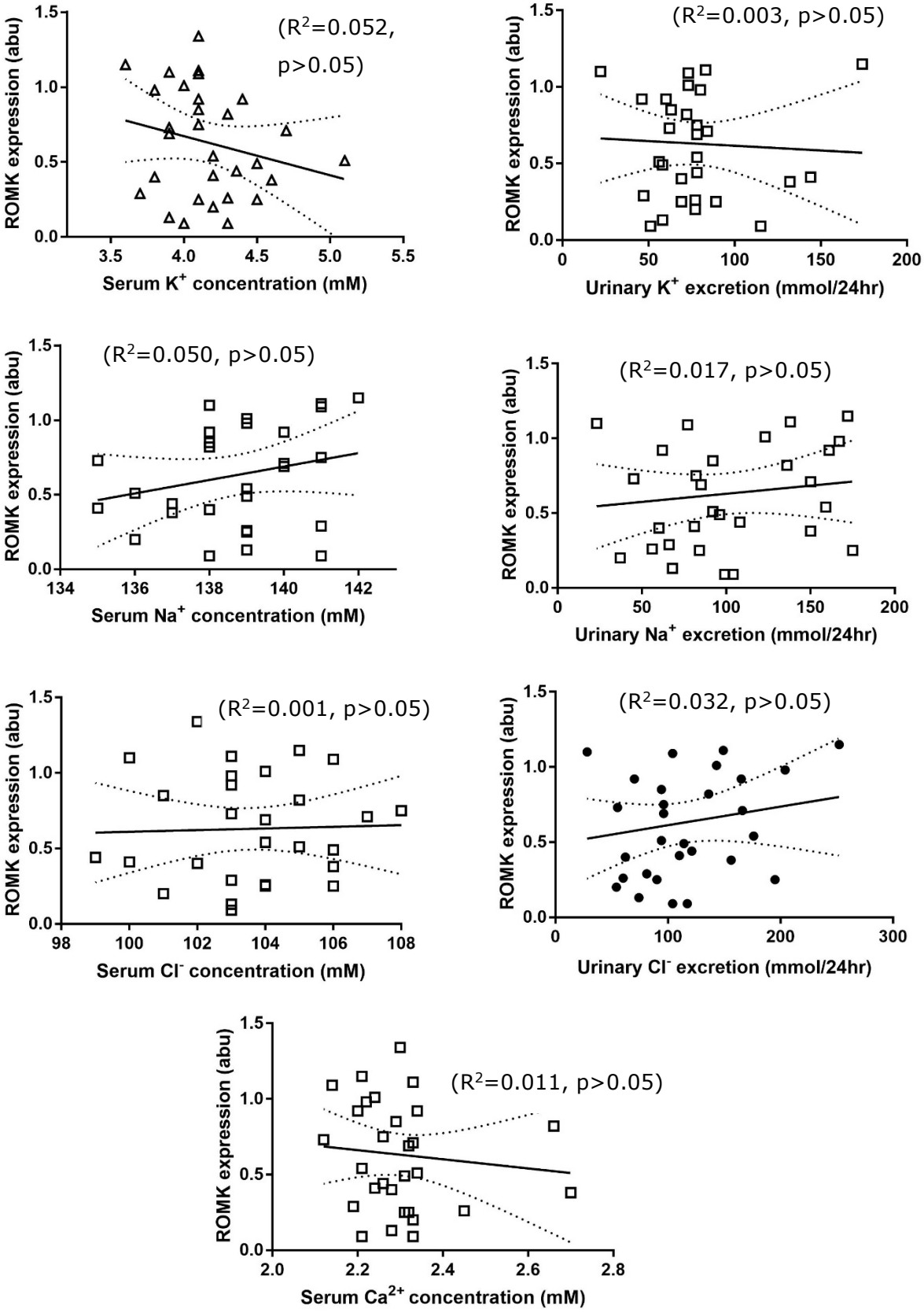


Figure 4-9 Linear correlation between ROMK and Serum K+ concentration, Serum Na+ concentration, Serum Cl- concentration, Serum Ca2+ concentration, 24hr Na+ excretion, 24hr K+ excretion, and 24hr Cl- excretion. *Each individual point represents a patient. Abu = Arbitrary units.*

4.4 Discussion

4.4.1 Serum and Dietary Potassium/Sodium

Murine studies by Terker *et* al. demonstrated that supra-physiological manipulations of dietary potassium intake and serum potassium concentrations regulate NCC [154]. This chapter set out to establish whether these correlations were seen in humans within normal ranges of serum electrolytes and dietary electrolyte intake.

Decreases in the expression of active NCC with increasing concentration of serum potassium have already been reported within rodent animal models and in patients with primary hyper-aldosteronism [116, 154]. In this chapter, data was presented which confirms correlations between pNCC and serum potassium in normonatraemic hypertensive patients. Serum potassium was correlated with pNCC S91 and T60 phosphorylation however; no significant correlation was seen with overall $NCC_{1/2}$ or NCC_3 expression. pNCC T55 also was not significantly correlated. However, the specificity of the pNCC T55 antibody could also target NKCC1/2 due to a close crossover (personal communication from Dr Glover), meaning the correlation observed for T55 may not truly represent the specific phosphorylation site against serum potassium. In addition to the reduced specificity of the T55 antibody, the differences in the correlations between phosphorylation sites could be due to variations in angiotensin II and aldosterone.

In this study, it was expected the 24hr urinary excretion and serum potassium would create a positive correlation, as dietary electrolytes are absorbed into the blood through the intestines, thus increasing concentration in the serum. As Figure 4-2 shows, no correlation was seen at all. An explanation for the absence of this specific correlation is that although the power calculation was used to identify the optimum number of samples in order to see an effect, more samples were needed for the expected positive correlation between serum potassium and urinary potassium excretion. Another explanation could be that in an otherwise healthy patient, serum potassium concentration is very tightly maintained across physiological dietary potassium intakes via mechanisms which don't involve NCC regulation.

An explanation for the lack of predicted correlations could be found in a study by Birukov *et,* al. where healthy men under conditions simulating prolonged confinement as may be experienced during manned travel to Mars [160]. It was concluded that there was no relationship between dietary electrolyte intake and 24h urinary excretion of electrolytes. This conclusion would explain the negative results of this chapter. Unfortunately, an exponential amount of work had been completed by the time this paper had been released [160].

In addition to the lack of correlation between 24hr urinary excretion and serum potassium, 24hr urinary potassium excretion did not correlate with pNCC S91 and T60, which contradicts previous rodent studies [165-167]. However, pNCC T55 positively correlated with dietary potassium, but upon looking closer at the spread of data, this correlation may be a false positive due to the 3 outliers in the higher urinary potassium excretion amount. A potential weakness of the study presented here is that in other human/mouse studies dietary potassium was directly measured. This study assumed that dietary potassium intake is reliably estimated by 24hr urinary excretion at a steady state. However, all other studies have measured dietary potassium by intake and so this is a potential limitation of this data. Estimation or measurement of dietary electrolyte intake is more challenging in humans than laboratory animals. However, regardless of whether it is serum or dietary potassium, there is a clear relationship between potassium and active NCC (pNCC). The data presented in this chapter also shows that serum potassium had more effect on pNCC than NCC_3 or $NCC_{1/2}$ expression.

Another study suggested an alternative mechanism, which may be important in the regulatory role of potassium. Ishizawa *et* al. proposed a low potassium diet increases NCC expression by stimulating the WNK-SPAK/OSR1-NCC pathway through KLHL3 [168]. With a low potassium diet, the phosphorylation of S433 of KLHL3 was increased, which leads to the suppression of the KLHL3/CUL3 ubiquitin ligase complex [168]. Whilst an increase in p-KLHL3 was observed, total KLHL3 decreased [168]. This change in expression leads to increased WNK4 expression and an increased NCC expression [168]. This study may offer an explanation for the observed correlations seen in this study.

Unlike other studies described above, in the study described in this chapter, potassium intake was not controlled in any of the patients, and no diet diaries

were taken making it difficult to identify those with high dietary potassium. By not controlling or recording potassium and other electrolyte intakes, the results are more representative of a patient's normal daily diet, giving a better physiological indication of exosomal protein expression.

With mouse model studies like in Terker *et* al., extremes of potassium intake can be investigated, but this is challenging for any human studies due to the extreme adverse effects i.e. hyperkalaemia and hypokalaemia [133, 154]. Perhaps the more important issue is the lack of face validity that such extreme electrolyte variation has on the ability to inform our understanding of the regulation of NCC over the physiological and pathophysiological ranges seen in human health and disease.

The advantage of not controlling potassium within patients' diets is that we are studying 'real life', as all drug-controlled hypertensive patients were assumed to have "normal" diets. Dietary K^+ intake (as estimated by 24hr urinary excretion) did not show any association with exosomal NCC unlike that reported by mouse model results published in the paper by Terker *et*, al. These differences could be explained by the extreme dietary K^+ intake used within the mouse models (0.05%-5% dietary K^+) [154], i.e. a 100-fold variation in dietary potassium intake in mice verus a 7-fold change in urinary potassium excretion seen in the patients of this chapter.

As no diet diary was given to patients in this study, it is impossible to report the type of potassium salts that were ingested. Various studies have shown that potassium salts vary the phosphorylation of NCC suggesting activation of the protein is dependent on the cation accompanying potassium e.g. chloride or bicarbonate [96, [169]. This may explain why dietary potassium did not correlate with NCC and related proteins as predicted.

4.4.2 Chloride's effect on NCC

Although this study aimed to focus on dietary and serum potassium effects on NCC, correlations between other electrolytes and NCC were observed. Serum chloride correlates negatively and significantly with NCC_3 expression, however the same cannot be observed for phosphorylated NCC or $NCC_{1/2}$. This difference in effect suggests that chloride may regulate the type 1 pathway of

NCC_3 only (Figure 4-1), rather than the type 2 pathway which is responsible for activating NCC with the addition of phosphorylation. As mentioned before (Section 1.11.3) chloride has inhibitory effects upon WNK4, which would be expected to lead to reduced expression of NCC. This could potentially explain the negative correlation seen in Figure 4-3 between chloride and NCC_3 expression.

Controversy remains as to the actual cause of the increase in NCC expression. Literature from over 30 years ago shows that chloride causes effects of hypertension only in combination with sodium [170, 171]. An animal study investigated oral administration of NaCl over several days and showed hypertensive effects within days, however when this was repeated with sodium-bicarbonate, the same effects were not observed [62]. These rat models showed the possibility that sodium alone has no effect on increasing blood pressure as previously thought. Other rat studies have concluded that the properties associated with a high halide diet (e.g. bromide or iodide) are more likely to increase blood pressure when comparing to a high sodium diet [172]. Early clinical studies in 1929 also demonstrated that a sodium-bicarbonate diet does not cause hypertension unlike NaCl [173]. The data presented in this chapter does not help in addressing this issue, as urinary chloride excretion did not positively correlate with any protein expression measured in this study.

4.4.3 Serum and dietary calcium and NCC

Evidence is emerging that calcium may contribute to the regulation of blood pressure through calcium-dependent proteins like calmodulin and calcineurin [63]. It is possible that these calcium-dependent proteins are responsible for the rapid dephosphorylation of NCC through signalling pathways currently unknown [63]. In recent studies, calmodulin was shown to rapidly dephosphorylate NCC indirectly through the regulation of calcineurin, which directly phosphorylates the cotransporter [63]. As these proteins only function in the presence of calcium, it is possible that calcium is a surrogate indicator of the activity in these proteins.

4.4.4 No WNK4 found in exosomes

WNK4 appears to be absent from the exosomes. This finding would suggest that WNK4 is exosomal independent or was subject to selective degradation, as active SPAK and NCC are expressed within the vesicles. However, Wolley *et*, al. presented a negative correlation between exosomal WNK4 and plasma potassium, although this study was focusing on the effects of mineralocorticoids on hypertensive patients [116]. Unlike Wolley's study, this chapter focuses on healthy hypertensive patients. This may suggest that WNK4 might be more heavily influenced by aldosterone and angiotensin II.

An alternative explanation would be that the level of WNK4 is too low to be seen with the antibody used, or that the antibody lacked specificity. However, without a positive control (e.g. an upregulated WNK4 cell line), it is impossible to conclude why WNK4 was absent from western blots in this study.

4.5 Summary

Murine studies have shown that supra-physiological variations in serum and dietary electrolytes affect renal NCC expression, however, there have been limited studies establishing whether this is reproducible in humans or in physiological ranges of electrolyte variation. This study is the first of its kind to report results in a hypertensive human population without hyperaldosteronism.

These initial human studies confirm serum potassium concentration correlates with phosphorylation changes in NCC. This suggests that serum potassium concentration is most important in modulating phosphorylating NCC (type II regulation), rather than changing the expression of NCC (type 1 regulation).

There is still uncertainty over the effect NaCl has on NCC, its related proteins, and blood pressure, as this study showed no positive correlations between the electrolyte and NCC expression. One suggestion is that increased concentrations of NaCl are linked to increased NCC expression, whilst another would be that Cl^- is the main effector of raised NCC. Further, more in-depth human studies are needed to be able to fully conclude this matter.

To gather conclusive data, further studies with the same population of patients, but with a controlled dietary potassium and sodium intake, would allow observation of NCC expression and phosphorylation status. It would also perhaps be of interest to collect data from patients taking no medication or before medication was started.

Chapter 5: Optimising the extraction of urinary exosomal mRNA

5 Optimising the extraction of urinary exosomal mRNA

Abstract

Exosomes have been reported in detail about the types of genetic material contained in the vesicles. However, despite many papers reporting the various types of miRNA present, there are very few detailing mRNA, especially in urinary exosomes. This may be due to the unstable nature of mRNA, making it increasingly difficult to extract the genetic molecule due to degradation.

The aim of this chapter is to extract mRNA from urinary exosomes using a variety of isolation methods from reported literature. Specific kits, SeraMir, MirCURY, ExoRNasy, ZR isolation, and GenElute, will be used to extract RNA from exosomes, after the exosomes have been isolated using ultrafiltration or chemical-isolation methods. The RNA samples extracted from the best methods will be outsourced for RNA-sequencing based on the sample quantity and quality, with the intention of identifying *SLC12A3* (NCC) or other regulatory mRNA transcripts. RNA sequencing will be validated by selecting genes of interest and performing qRT-PCR.

Combining the data collected from validation (RiboGreen and Bioanalyzer assays), RNA-sequencing FPKM values and qRT-PCR expression results, it can be concluded that SeraMir was the best exosomal mRNA extraction method, followed by MiRCURY. However, overall the quality and quantity of RNA extracted was lower than usually expected for RNA sequencing. Although *WNK1* and *CUL3* were successfully used to validate qRT-PCR, *SLC12A3* (NCC) and other related genes were not expressed. For future experiments, RNase inhibitor should be used to reduce the level of exosomal degradation.

5.1 Introduction

Messenger RiboNucleic Acid (mRNA) is the 'stepping stone' between DNA and protein coding, as well as vital to the regulation of cells through non-coding sub-species e.g. microRNA (miRNA). mRNA is transcribed from DNA within the nucleus, before travelling into the cytoplasm where it is translated into proteins by a tRNA/ribosome complex. In addition to proteins, exosomes are known to contain RNA in particular miRNA [174]. miRNA has been extensively studied within exosomes, as they are stable and present in relatively high concentrations, making them excellent potential biomarkers for disease and suggesting they may play a role in cell regulation [175, 176]. mRNA, expression profiles are helpful as they give insight into gene expression in a cell at a given time, which is information not gained from sequencing the whole genome [177]. However, mRNA is less stable than miRNA, and hence analyses may lead to smaller and sometimes un-readable RNA sequences [178]. There are very few studies, which have worked with mRNA in exosomes due to this fragility and a high potential for degradation. This chapter aims to describe work to develop the best methodology for extracting mRNA from exosomes using current literature as its starting point, with the aim of sequencing RNA from exosomes derived from samples of human urine.

Various literature has described an assortment of RNA extractions from exosomes: SeraMir, MirCURY, ExoRNasy, ZR isolation, and GenElute [179-182]. These kits are the most commonly used methods of exosome RNA extraction, each having their advantages and disadvantages. Table 5-1 describes these points. It is important to establish which method results in the best quality and quantity mRNA for human urinary exosomes.

Table 5-1 Advantages and Disadvantages of Extraction Methods for RNA. *Below lists the 5 exosomal RNA extraction methods used, which were selected from the literature* [179-184].

Extraction Methods	Advantages and Disadvantages
Seramir	• Better qPCR Profiling • Simple to use • Quick • Efficient • Specific to exosomes
	• Increased chance of Long-non-coding RNA (lncRNA)
MirCURY	• Specific for extracellular vesicles • Easy to use
	• Not RNA exosome specific
ZR isolation	• Specific to urinary exosomes • Easy • Reliable • Rapid isolation
	• High chance of protein contamination
ExoRNasy	• Extracellular vesicles RNA specific • Easy to use • Not technically challenging
	• Requires precipitation solutions for best results
GenElute	• Simple • Efficient • High yield from mammalian cells
	• Not exosome RNA specific • More technically demanding • High chance of contamination

Previous work has shown the stability of all RNA species are significantly higher within the exosomes than when released, and this is particularly true with mRNA [185]. Within the exosomes, mRNA is surrounded by buffers, which help maintain the sequence and structure, and which must be re-created within extraction methods, i.e. by the use of DNase and RNase inhibitors [181, 185]. By extracting mRNA from urinary exosomes, this provides the greatest chance of analysing kidney and bladder biology non-

invasively. Free urinary RNA is significantly less stable due to its exposure to DNAse and RNAse enzymes and other destructive molecules. However, by maintaining RNA within the exosomes for as long as possible, the possibility of RNA degradation is reduced due to decreased exposure to RNase enzymes

As mentioned above, the aim of this chapter is to extract mRNA from exosomes using a variety of isolation methods. The RNA samples extracted from the best methods will be outsourced for RNA-sequencing based on the sample quantity and quality, with the intention of identifying *SLC12A3* (NCC) or other regulatory mRNA transcripts.

In this chapter, I performed all exosomal and RNA extraction from the 11 extraction methods detailed below. Before RNA samples were sent to Oxford Genomics for RNA sequencing, I also measured the RNA quantity and quality by RiboGreen assays (see Section 2.6.3). Once RNA sequencing was complete, Oxford Genomics sent the raw fastq data files generated by the HiSeq4500 machine. All data analysis was performed in-house by myself.

5.2 Results

As discussed above, there are many RNA materials and reagents, which can be used to extract RNA from exosomes, following the extraction of exosomes from urine samples. In order to identify the best RNA extraction method, 11 various methods were chosen to combine our own methods and those described in the literature. Two types of exosome extractions were used: Ultrafiltration and exosome binding reagents. RNA was extracted from exosomes using 5 different methods from kits from different companies. With the combination of exosome extraction and RNA extraction, each method was given their own code for easy reference. 10 healthy volunteers donated urine, which was split equally between each of the 11 extraction methods (except GP, MG and EM). Below is a summary of the sample names and their extraction process:

Table 5-2 Sample names and their RNA extraction process. *[1]describes exosomal extraction; [2]describes mRNA extraction*

Sample names	Exosome extraction process
S1 - S10	UF[1] + SeramiR[2]
M1 - M10	UF [1] + mirCURY™ [2]
E1 - E10	UF[1] + ExoRNeasy® [2]
Z1 - Z10	UF[1] + ZR isolation™ [2]
G1 - G10	UF[1] + GenElute™ [2]
SP1 - SP10	Exoquick[1] + SeraMir[2]
EP1 - EP10	exoEasy® [1] + exoRNeasy® [2]
MP1 - MP10	mirCURY™ [1] + mirCURY™ RNA[2]
GP1 - GP5	exoEasy® [1] + GenElute™ [2]
MG1 - MG5	mirCURY™ [1] + GenElute™ [2]
EM1 - EM5	exoEasy® [1] + mirCURY™ RNA[2]

Following extraction, quality control tests were performed to identify which extraction methods produced the mRNA suitable for RNA-sequencing.

5.2.1 Nanodrop, Ribogreen and Bioanalyzer Validation

Urine samples used were collected from non-hypertensive normonatraemic controls and treated with protease inhibitor. Samples were stored at -80°C for up to 1 month as described in Section 2.2. The best extraction method was chosen based on results from Nanodrop, Ribogreen assays and Bioanalyzer, providing maximum information about specificity and sensitivity.

5.2.1.1 Nanodrop

The Nanodrop assay (a simple and time-efficient method) calculated the nucleic acid purity (260/230) and DNA to RNA ratio (260/280) as well as the concentration of RNA and DNA. Nanodrop gives an early indication of any contaminants. Concentrations determined by the Nanodrop showed the GP method produced the highest average concentration: 74.72±41.61ng/µL, whilst the G method produced the lowest concentration 6.47±1.90ng/µL. However, Nanodrop pictographs presented with high peaks within the protein range (280nm). It was concluded results from the Nanodrop were false positives and therefore must be excluded when deciding the optimal method for extracting RNA.

Figure 5-1 shows an example of a Nanodrop Pictograph from samples extracted with Seramir. The remainder of the pictographs can be seen in the Appendix.

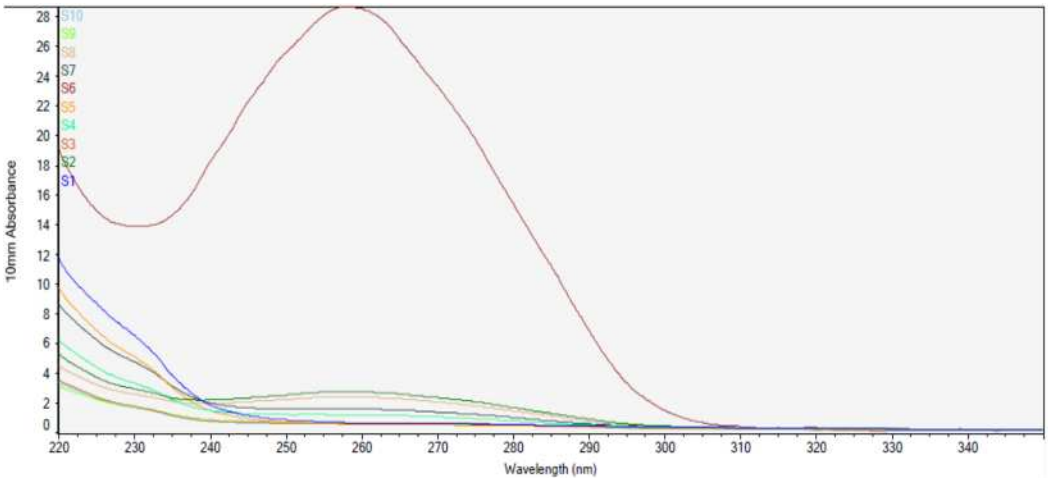


Figure 5-1 Nanodrop pictograph for Seramir extraction method. *Other extraction methods can be seen in the Appendix*

5.2.1.2 Ribogreen

Ribogreen assays specifically measure mRNA only, unlike the Nanodrop, which measures all nucleic acids. Initial results of the Ribogreen assay showed that the Seramir (S1-10 samples) extraction produced the highest concentration of mRNA. Statistical analysis from this assay shows S samples (22.32ng/µl±18.13) have a significantly higher ($p<0.01$) mRNA concentration than any other of the 11 groups. The Ribogreen assay detected no RNA concentrations from samples extracted from GenElute extractions (G samples), which was also observed with the Nanodrop quantification.

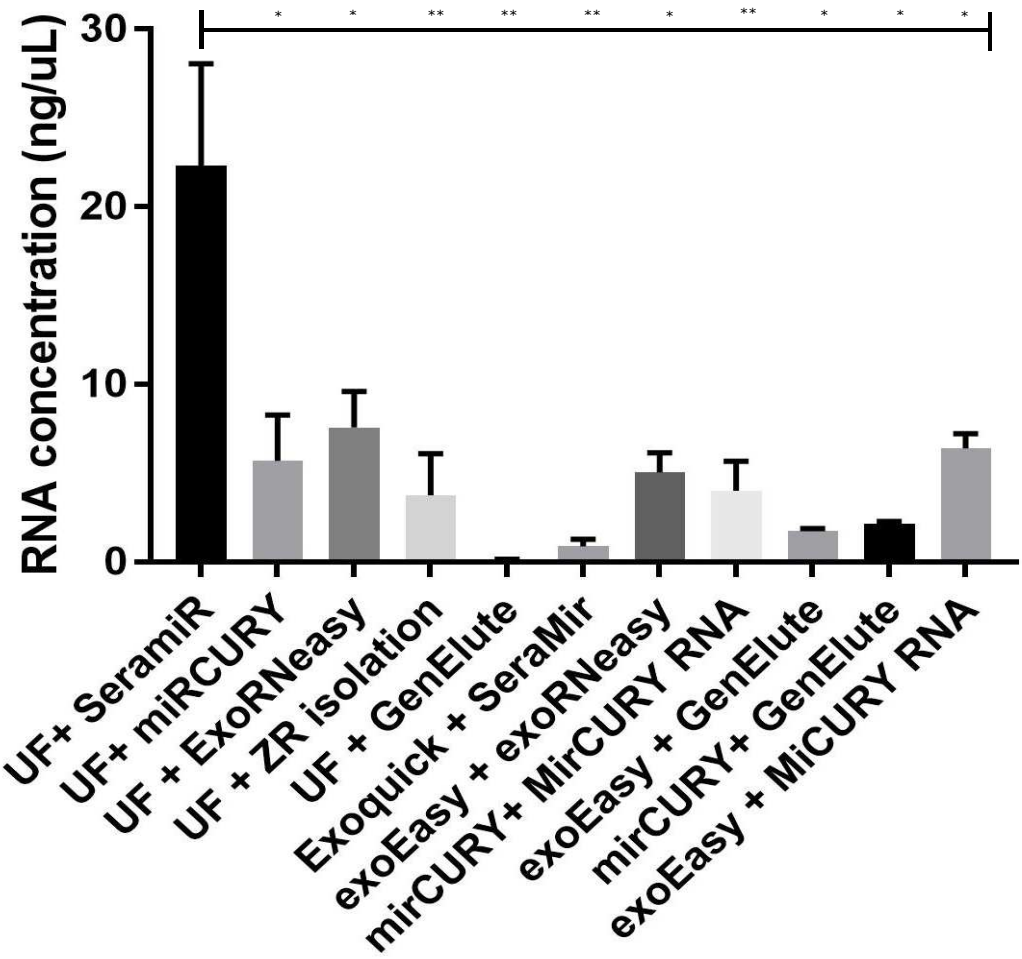


Figure 5-2 Average concentration (with SEM) of each extraction method samples using Ribogreen assays. *One-way Anova showed significance against UF + SeramiR and the other methods (*, $p<0.05$; ** $p<0.0001$). Other comparisons between groups showed no statistical significance ($p>0.05$.*

5.2.1.3 Bioanalyzer RNA 600 Pico Assay

As with Ribogreen assays, the results from the Bioanalyzer RNA 600 Pico assay showed the Seramir extraction (S samples) yielded the highest mRNA concentration (3605.35pg/µl±6736.18) when compared to the other 11 different extraction methods. The Bioanalyzer RNA kit showed a lower concentration than that of Ribogreen assays, most likely due to the increased specificity of the Bioanalyzer. As with Nanodrop and Ribogreen assays, the GenElute extraction (G samples) produced the lowest concentration (127.90 pg/µl±70.58) when using the Bioanalyzer.

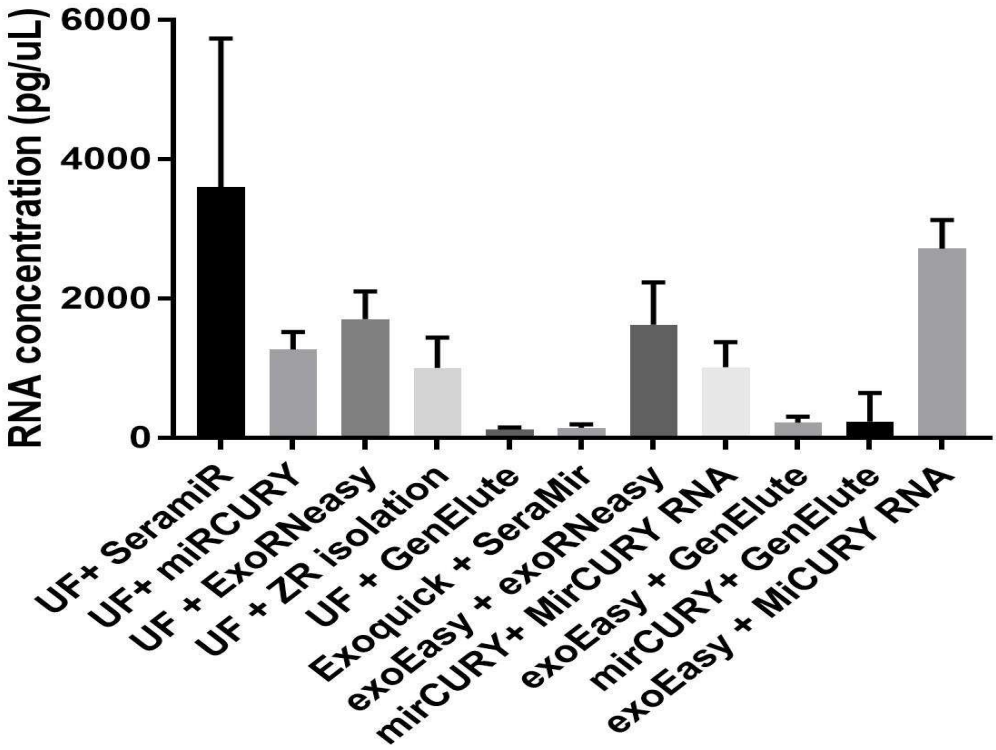


Figure 5-3 Average concentration (with SEM) of Extraction Method samples from the Bioanalyzer RNA 6000 Pico Assay. *Anova test showed no significant difference between the 11 extraction methods produced p>0.05.*

Table 5-3 Nanodrop, Ribogreen and Bioanalyzer concentrations of RNA extraction method. *Average RNA concentration with their standard deviation and SEM are included for each quality control test. The results show displays the values of each validation method. Each extraction method includes the average of RNA concentration, Standard Deviation (SD) and Standard Error of Mean (SEM). The Nanodrop validation includes the 260/280 (DNA to RNA ratio) and 260/230 nucleic acid purity), proving this particular validation method produced false positives. Bioanalyzer includes "mRNA region" to show the percentage of mRNA within each sample.*

Samples		Nanodrop			Ribogreen	Bioanalyzer	
		Conc (ng/µl)	260/280	260/230	Conc (ng/µl)	Conc(pg/µl)	mRNA region
S1-S10	$\bar{x}$	48.27	1.57	0.55	22.32	3605.35	53.5
	SD	39.05	0.19	0.61	18.13	6736.18	39.38
	SEM	12.35	0.06	0.19	5.73	2130.17	12.45
M1-M10	$\bar{x}$	38.84	3.67	0.30	5.71	1271.9	23.8
	SD	14.13	1.13	0.12	8.14	799.77	13.19
	SEM	4.47	0.36	0.04	2.57	252.91	4.17
E1-E10	$\bar{x}$	42.97	1.34	0.17	7.58	1709.5	36.4
	SD	15.99	0.39	0.06	6.46	1258.30	28.89
	SEM	5.06	0.12	0.02	2.04	397.91	9.14
Z1-Z10	$\bar{x}$	16.48	3.69	0.33	3.75	1008.00	35.70
	SD	11.14	2.23	0.35	7.50	1379.76	21.05
	SEM	3.52	0.71	0.11	2.37	436.32	6.66
G1-G10	$\bar{x}$	6.47	2.26	0.15	-0.22	127.90	16.30
	SD	1.90	0.15	0.07	0.53	70.58	7.10
	SEM	0.60	0.05	0.02	0.17	22.32	2.25
SP1-SP10	$\bar{x}$	38.19	2.31	0.47	0.90	146.30	37.30
	SD	5.01	0.60	0.17	1.23	163.84	9.36
	SEM	1.58	0.19	0.05	0.40	51.81	2.96

Table 5-4 continued…

Samples		Nanodrop			Ribogreen	Bioanalyzer	
		Conc (ng/µl)	**260/280**	**260/230**	**Conc (ng/µl)**	**Conc(pg/µl)**	**mRNA region**
EP1-EP10	$\bar{x}$	48.62	2.38	0.13	5.07	1625.70	35.40
	SD	9.99	3.27	0.03	3.43	1933.76	26.85
	SEM	3.16	1.03	0.01	1.08	611.51	8.49
MP1-MP10	$\bar{x}$	32.55	2.483	0.251	4.02	1019.20	40.50
	SD	6.64	0.60	0.07	5.30	1126.16	14.51
	SEM	2.10	0.19	0.02	1.68	356.12	4.59
GP1-GP5	$\bar{x}$	74.72	1.87	1.29	1.76	222.80	52.40
	SD	41.61	1.04	0.74	0.39	261.08	13.43
	SEM	13.16	0.33	0.23	0.12	82.56	4.25
MG1-MG5	$\bar{x}$	6.64	0.167	0.13	2.15	236.20	94.60
	SD	4.41	0.11	0.13	0.42	1298.35	9.28
	SEM	1.39	0.03	0.04	0.13	410.57	2.94
EM1-EM5	$\bar{x}$	47.54	1.18	0.68	6.42	2723.20	20.80
	SD	6.56	0.16	0.12	2.56	1298.35	9.28
	SEM	2.08	0.05	0.04	0.81	410.57	2.94

5.2.2 Sequencing of exosomal RNA

Using the validated methods and the data collected from RiboGreen assays and the Bioanalyzer, 4 extraction methods were selected for RNA-sequencing: Seramir (S), MirCURY (M), ExoRNasy (E), and Exoquick/MirCURY (EM). Once the extraction methods were chosen, specific samples were selected for RNA sequencing. The selection was made based on the individual sample's quality and quantity of RNA, measured from the RiboGreen and Bioanalyzer data. Of these top 4 extraction methods, 10 samples and 2 blank samples were selected for RNA sequencing (3 samples from S method, 3 samples from M method, 2 samples from EM method and 2 samples from the E method). The number of samples chosen for RNA sequencing was based on economic reasons.

As the Nanodrop produced false positive results; therefore, selecting the best methods were made from concentrations generated by Ribogreen and Bioanalyzer assays. Once normalised to 40uL, the samples were sent in a 96-well plate on dry ice to Oxford Genomics, Oxford for RNA sequencing. As the samples were low in quality, Oxford Genomics concentrated the samples using AMPure beads (See Section 2.8.1). During library prep, one sample from the Seramir method had to be removed, as the sample was too poor quality to continue sequencing. Figure 5-4 shows the concentration at submission and after concentration using AMPure beads (data collected by Oxford genomics).

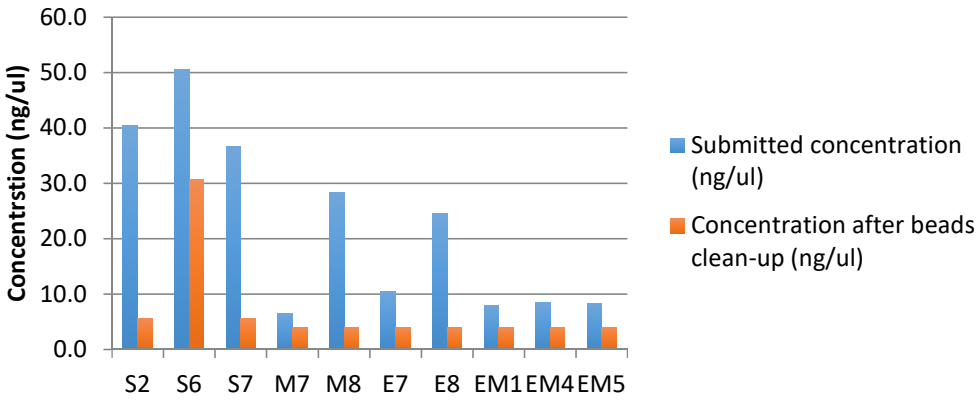


Figure 5-4 Bar chart to show the comparison of the concentration of RNA before and after using AMPure beads. *As samples showed a low quality and RIN score, Oxford Genomics advised on concentrating the samples using AMPure beads. As can be seen, most samples are 5ng/µL or below. Data collected and produced by Oxford genomics.*

Figure 5-5 shows Oxford genomics Bioanalyzer data. RNA samples with RIN scores between 1.0-1.8 and fragments below 500bp are shown. Due to the small fragment sizes, RNA of 150-250bp was selected to minimize sequencing miRNA.

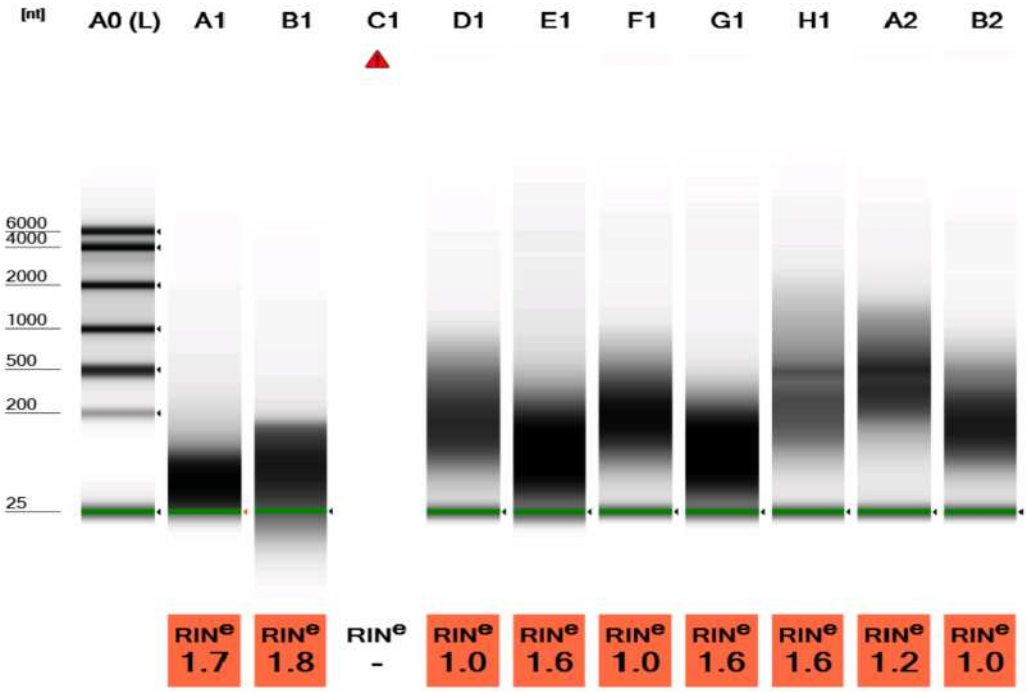


Figure 5-5 Bioanalyzer Gel images showing the distribution of mRNA lengths. *In total, the base pair lengths are very low (<200bp). Lane C1 (sample S6) failed, due to lack of quality. The result was given by Oxford Genomics.*

Once RNA raw fastq data files were received from Oxford Genomics, quality control checks were required after collating files before identifying Fragments Per Kilobase of transcript per Million (FPKM) values from sequence analysis. Chapter 2.8.3.2 describes the graphs' purpose and the quality of reads for every sample.

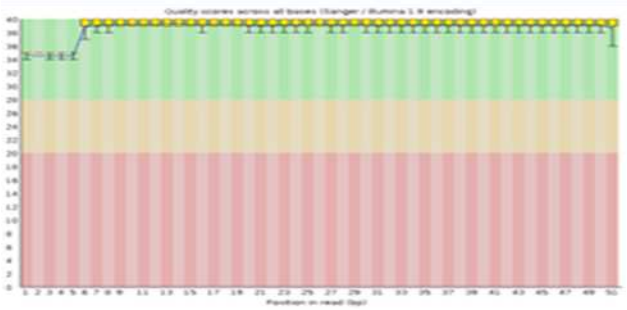
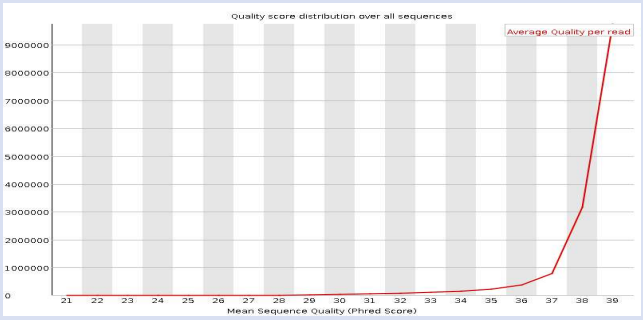
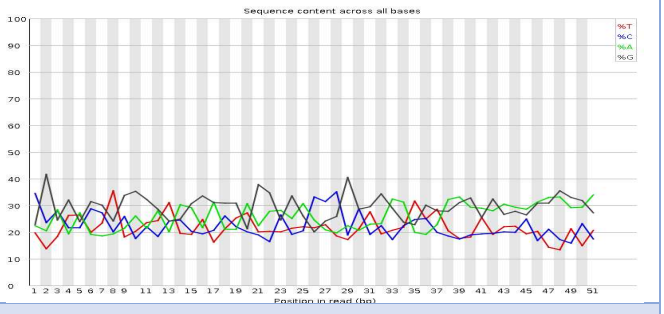
5.2.3 Expression of *SLC12A3* and related genes

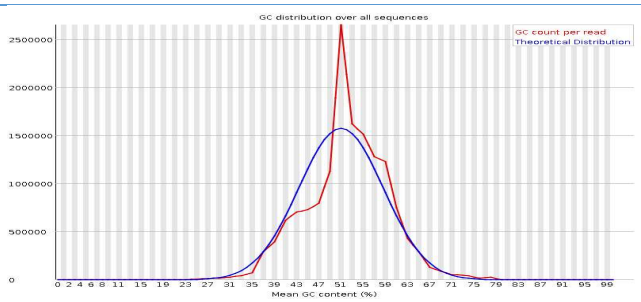
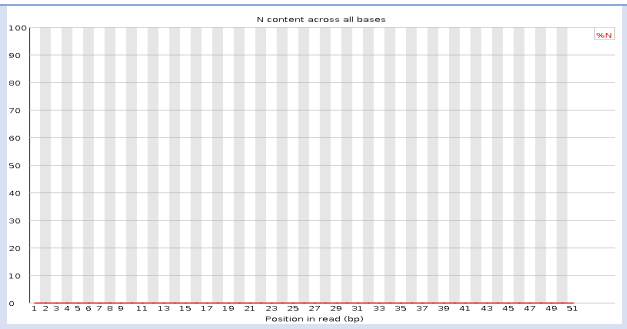
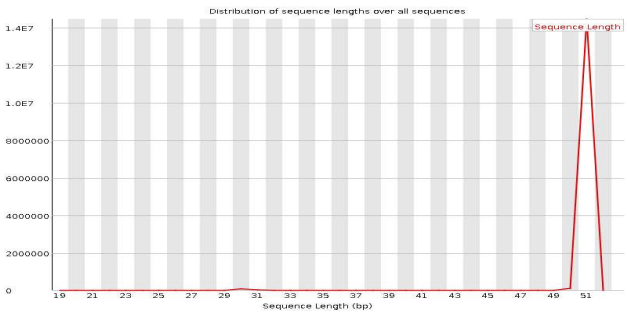
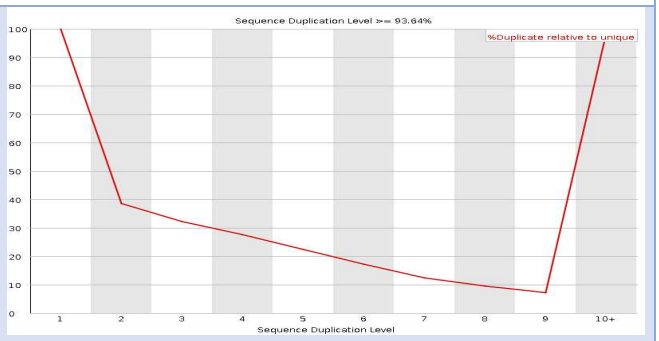
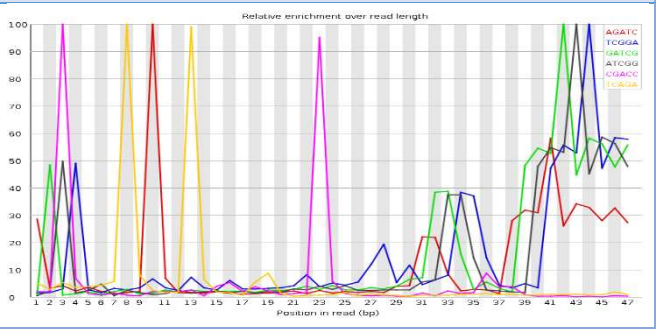
After receiving the raw data (fastq files) from Oxford Genomics, the data was collated into one file. Each file generated a read number of $2.15x10^8$ to $3.76x10^8$.

5.2.3.1 Quality control Checks

Table 5-5 shows representative quality control data for RNA sequencing of a single sample (in this instance, sample S2). Quality control data was generated using the FASTQC report software. Graphs of most importance include "per base sequence quality", "Per Base Sequence Content", "Per Base GC Content", and "Per Sequence GC Content". These graphs were used to decide on the continuation of RNA sequencing analysis. All samples produced acceptable quality control results, therefore analysis continued to merging sample files with the published Human Genome (TopHat programme).

Table 5-5 Sample S2 (Seramir RNA extraction method) FastQC Report. *Graphs and data shown were used to decide the continuation of RNA-sequencing. Graphs of most importance are "per base sequence quality", "Per Base Sequence Content", "Per Base GC Content", and "Per Sequence GC Content".*

FastQC module	Function
Basic Stats	Total Sequences: 14888475 Filtered Sequences: 0 Sequence length: 20-51 %GC: 52
Per Base Sequence Quality	
Per Sequence Quality Scores	
Per Base Sequence Content	
Per Base GC Content	

Per Sequence GC Content	
Per Base N Content	
Sequence Length Distribution	
Duplicate Sequences	
Overrepresented Kmers	

5.2.3.2 Alignment to the Human Genome

After quality control, TopHat was used to begin with the alignment of samples with the published Human Genome v37 (Section 2.8.3.3). Alignment commands were used to show the percentage of each sample aligning with the Human Genome.

Table 5-6 Alignment data from samples sequenced. *These data describe how much of the overall human genome is represented in each sample and how much of the sample contains repeated fragments.*

Samples	Mapped input	Multiple Alignments
E7	29.6%	9.4% (3435 have >20)
E8	55%	8.2% (12613 have >20)
EM1	26%	40.9% (10435 have >20)
EM4	9.6%	20.6% (1465 have >20)
EM5	26.7%	23.1% (8354 have >20)
S2	33.6%	9.6% (26720 have >20)
S7	21.4%	6.9% (5451 have >20)
M7	34.8%	7.9% (5270 have >20)
M8	53%	6.6% (13463 have >20)

The command also shows how much of the sample contains repeated sequences (multiple alignments). Table 5-6 shows the EM extraction method to have a high amount of multiple alignments (23.1-40.9%), indicating that a large proportion of the sample has repeated sequences. MiRCURY extraction method (M7 and M8) aligned closest to the Human Genome when compared to the other extraction methods (34.8 and 53%, respectively). MiRCURY also had the fewest multiple alignments (6.6-7.9%). Overall, the mapped input was low, with only a few samples reaching over 50% (E8 and M8).

Due to the nature of using exosomes, it is likely there was a high amount of degradation of mRNA from the point of exocytosis to the extraction of RNA. It could be speculated that exosomes also can be selective in the mRNA that is excreted, as seen with miRNAs.

Table 5-7 List of Candidate genes with established function in distal nephron sodium or water trafficking and their FPKM numbers in each sample.

Genes	E7	E8	EM1	EM7	EM8	M7	M8	S2	S7
SLC12A3	1.00	0.52	0.00	3.87	4.67	0.86	1.27	3.88	0.92
PDCD6IP **(ALIX)**	0.51	9.36	1.28	4.40	18.05	17.69	3.95	27.55	10.59
AQP2	0.00	0.43	0.00	0.00	2.77	4.46	0.17	3.18	3.67
SLC12A1 **(NKCC2)**	1.55	1.86	0.76	119.86	0.00	4.23	1.42	7.31	8.76
SLC12A2 **(NKCC1)**	6.63	0.75	8.53	2.42	1.26	0.00	2.43	2.62	0.90
SCNN1A **(ENaCA)**	2.78	5.09	8.21	1.99	7.27	11.59	5.18	15.00	48.95
SCNN1B **(ENaCB)**	0.87	0.96	0.00	9.81	1.89	12.90	2.85	4.49	34.59
SCNN1D **(ENaCD)**	1.27	0.80	0.00	2.45	0.00	3.10	6.37	0.00	4.70
SCNN1G **(ENaCG)**	4.05	4006.57	0.00	0.00	1.05	3.20	3.74	3.51	7.69
WNK1	15.09	9.61	19.23	48.14	19.68	29.34	16.60	14.84	39.94
WNK2	1.53	4.45	2.25	15.70	3.98	2.09	2.02	19.24	10.86
WNK3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
WNK4	0.30	0.32	6.36	0.00	0.62	0.64	27.93	0.70	1.03
KLHL3	2.52	4.24	4.38	0.00	0.98	6.57	12.47	0.40	6.47
CUL3	33.36	26.98	28.47	1.67	22.36	37.29	72.17	54.54	34.15
KCNJ1 **(ROMK)**	0.00	0.08	0.00	0.00	0.45	0.74	2.09	2.32	1.05
SLCO2A1 **(PGT)**	0.00	0.68	0.00	0.00	0.00	0.00	0.61	0.28	6.15
GAPDH	20.10	11.72	11.63	5.35	11.54	53.71	6.51	86.04	103.82
β-actin **(ACTB)**	141.58	23.52	133.95	99.05	73.30	282.38	25.43	389.02	795.68
SLC9A3 **(NHE3)**	0.91	3.56	0.00	0.00	0.00	2.82	8.75	0.00	4.21
CFTR	5.22	40.13	0.00	0.00	81.84	3.88	2.61	0.00	0.00

Table 5-8 Top 20 genes identified through Cuffnorm RNA sequencing analysis. **** See Table 5-9*

Rank	E7	E8	EM1	EM7	EM8
1	*RP11-463I20.3*	*RNA5-8SP6*	*MB21D1*	*CTD-2328D6.1*	*CTD-2328D6.1*
2	*RPL24P2*	*TRPM6*	*CTD-2328D6.1*	*RPPH1*	*RNA5SP481*
3	*Y_RNA*	*ELF2*	*RNA5SP481*	*MT-TE*	*MT-TE*
4	*RASSF8*	*CASC11*	*MT-TS1*	*RNA5SP481*	*MT-TS1*
5	*CTD-2328D6.1*	*CTD-2328D6.1*	*SNHG1*	*MT-TA,MT-TC,MT-TN,MT-TY*	*5S_rRNA*
6	*DUX4L18*	*DUX4L18*	*FCGR2A,FCGR2B,FCGR2C, RP11-25K21.6*	*FCGR2A,FCGR2B,FCGR2C, P11-25K21.6*	*RNA5SP242*
7	*RNA5-8SP6*	*TPH1*	*MT-TA,MT-TC,MT-TN,MT-TY*	*GNB2L1,SNORD95,SNORD96A*	*SNHG1*
8	*SNHG1*	*AC096643.1*	*5S_rRNA*	*MT-TS1*	*RPL24P2*
9	*TRPM6*	*MCC*	*RNA5SP242*	*DUX4L18*	*RPPH1*
10	*LRRC4C*	*RP11-401O9.4*	*MT-TE*	*SNHG1*	*RNA5SP74*
11	*GPR39*	*NEDD4*	*EIF4A2,SNORA4,SNORA63, SNORA81,SNORD2*	*RNA5-8SP6*	*GAS5*
12	*MAP2K4P1*	*****	*GNB2L1,SNORD95,SNORD96A*	*RNA5SP242*	*RNA5-8SP6*
13	*RP11-12M5.3*	*SLU7*	*RNA5SP74*	*****	*GNB2L1,SNORD95, SNORD96A*
14	*PPP1R21*	*INTS7*	*RPL23A,SNORD42A, SNORD42B,SNORD4B*	*RNY5*	*RPL23A,SNORD42A, SNORD42B,SNORD4B*
15	*AP001604.3*	*MAVS*	*SNHG5*	*5S_rRNA*	*MT-TA,MT-TC,MT-TN, MT-TY*
16	*RALGAPA2*	*TXLNB*	*RNA5-8SP6*	*FAM211A-AS1*	*****
17	*C5orf42*	*RP11-932A10.1*	*SNORD99*	*RNY4*	*ARID2*
18	*CTA-714B7.5*	*RALGAPA2*	*RPS8,SNORD38A, SNORD38B,SNORD55*	*RPL23A,SNORD42A, SNORD42B,SNORD4B*	*AC010970.2*
19	*CASC11*	*ITM2C*	*RNY5*	*RNY1*	*SNHG5*
20	*RP11-335I12.2*	*HPCAL1*	*RNY4*	*RNA5SP74*	*FCGR2A,FCGR2B,FCGR2C, RP11-25K21.6*

Table 5-9 Top 20 genes identified through Cuffnorm RNA sequencing analysis continued. *** *MT-ATP6,MT-ATP8,MT-CO1,MT-CO2,MT-CO3,MT-ND1,MT-ND2,MT-ND3,MT-ND4,MT-ND4L,MT-ND5,MT-RNR1,MT-RNR2,MT-TF,MT-TG,MT-TH,MT-TI,MT-TK,MT-TL1,MT-TL2,MT-TM,MT-TR,MT-TS2,MT-TV,MT-TW*

Rank	M7	M8	S2	S7
1	*RNA5-8SP6*	*RNA5-8SP6*	*RNY1*	*NIPAL3*
2	*CTD-2328D6.1*	*TRPM6*	*CTD-2328D6.1*	*RNA5-8SP6*
3	*RPL24P2*	*CTD-2328D6.1*	*RPPH1*	*CTD-2328D6.1*
4	*AC010970.2*	*ELF2*	*RNY4*	*IL18R1,IL1RL1*
5	***	*INTS7*	*MT-TE*	*MT-TE*
6	*TRPM6*	*DUX4L18*	***	***
7	*SART3*	*NEDD4*	*RNA5-8SP6*	*GNB2L1,SNORD95,SNORD96A*
8	*MT-TE*	*SNHG1*	*AC010970.2*	*SNHG1*
9	*SNHG1*	*TPH1*	*SNHG5*	*RPS8,SNORD38A,SNORD38B,SNORD55*
10	*DUX4L18*	*CASC11*	*SNHG1*	*AC010970.2*
11	*MT-TS1*	*RALGAPA2*	*Y_RNA*	*RNY1*
12	*RN7SL1*	*UPK2*	*MT-TA,MT-TC,MT-TN,MT-TY*	*SNHG5*
13	*SNHG5*	*MGRN1*	*RNY5*	*RNY4*
14	*RALGAPA2*	*SFMBT2*	*Y_RNA*	*MT-TS1*
15	*RN7SL2*	*RP11-358H9.1*	*SNORD63*	*DUX4L18*
16	*THBS4*	*OTUD5*	*RN7SL1*	*RNA5SP481*
17	*RNY4*	*MAVS*	*FCGR2A,FCGR2B,FCGR2C, RP11-25K21.6*	*RN7SL2*
18	*MGAT4A*	*AC096643.1*	*RN7SL2*	*RN7SL1*
19	*MIRLET7DHG*	*GAS5*	*SNORD71*	*CASC11*
20	*MCC*	***	*GAS5*	*KRT13*

Table 5-7 describes the FPKM values of genes of interest in this thesis. Most candidate genes were considered as not expressed due to the low FPKM values. The threshold value was selected at 10, due to the low levels of expression. As expression was above the threshold, *WNK1* and *CUL3* were selected as genes of interest for qRT-PCR validation. *β-actin* was expressed higher than the threshold, but as it was not found in the top 20 most highly expressed, it was considered not optimal for a house-keeper gene. In sample E8, the gene *SCNN1G* can be concluded as an error, due to its FPKM value (FPKM=4006.57) showing as 20x higher than β-actin (FPKM=23.52).

Of those genes of interest that were expressed, SeraMir extraction and MiRCURY extraction methods produced the highest FPKM values.

Table 5-8 and Table 5-9 display the top 20 genes across all samples sent for sequencing (referenced to the Human Genome version 37). This list was used to assess high levels of expression for qRT-PCR validation studies and used compare against genes of interest e.g. *SLC12A3*, *WNK1* and *CUL3*. The two most common genes to appear were selected: *RACK1* (Receptor For Activated C Kinase 1) and *TRPM6* (Transient Receptor Potential ion channel).

5.2.4 Validating RNA-sequencing with qRT-PCR

To validate the data generated from RNA-sequencing analysis, qRT-PCRs were performed. cDNA synthesis was performed on all RNA samples from the 11 extraction methods described above. All RNA samples were normalised to at 5ng/μL (Section 2.7.2). With all qRT-PCR runs, a Reverse Transcriptase (RT) negative and No Template Control (NTC) sample were included.

5.2.5 *RACK1* and *TRPM6* expression using Taqman primers

Genes that appeared frequently (more than twice) within the top 20 table (Table 5-8 and Table 5-9) were selected as house-keeper genes. As a result, *RACK1* (known as *GNB2L* in Table 5-8 and Table 5-9) and TRPM6 were chosen to target the remaining samples left. Usual house-keepers (reference genes), *GAPDH* and *β-actin*, were present below the chosen threshold FPKM values and therefore could not be used in this experiment.

5.2.5.1 Optimisation with control Fetal Kidney Total RNA

To ensure that the *RACK1* and *TRPM6* primers worked in a control sample, total RNA from Fetal Kidney (Agilent) was used as a positive control. The results showed strong expression with *RACK1*, but little or no expression of *TRPM6*. The total RNA was aliquoted into serial dilutions and plated in triplicates, with the average Cyclic threshold (Ct) value taken. For future qRT-PCR experiments, the optimal concentration for the positive control was 1:100.

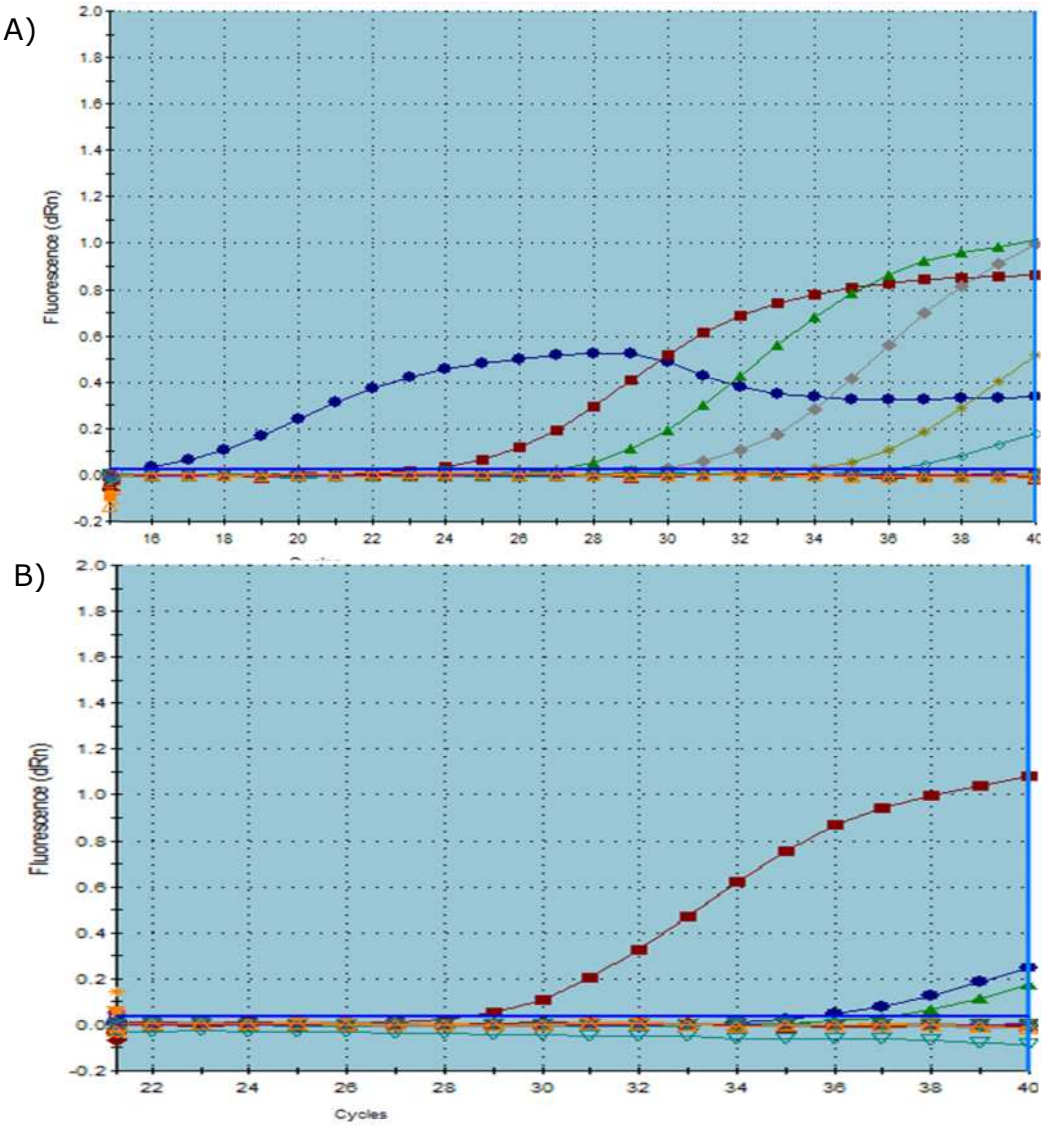


Figure 5-6 Serial Dilution of RACK1 and TRPM6 Amplification plots and Ct values of Total Fetal Kidney RNA. *The PCR protocol ran for 40 cycles. A) With RACK1, the neat sample (highest concentration) was detected after 16 cycles. B) TRPM6 was not highly expressed after 40 cycles, with the highest concentration detected after 33 cycles. RT-ve was blank, producing no Ct value, as expected.*

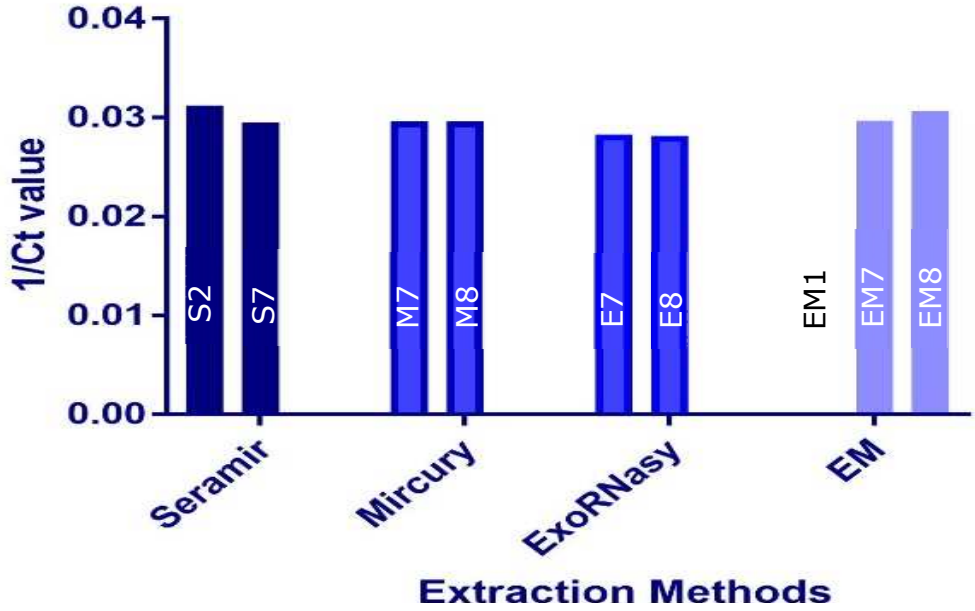
5.2.6 *WNK1* and *CUL3* are the highest expressed candidate genes within urinary exosomes

Once *RACK1* had been established as the house-keeper gene for these experiments, genes related to this project were selected from FPKM values. *CUL3* and *WNK1* were the only genes with high enough values and selected for validation experiments.

Although the FPKM values for *SLC12A3* (NCC) were negligible, qRT-PCR experiments were performed, as the gene product is the target of thiazides. As expected, no Ct values were obtained, and so further analysis of the mRNA/gene was not pursued.

5.2.6.1 CUL3

To compare expression between extraction processes, raw Ct values were evaluated. In graphs, Ct values were presented as 1/Ct value. The expression values (Ct values) were similar for each extraction methods. Looking at qRT-PCR results alone, it could be concluded that EM1 does not express *CUL3*; however, EM1 FPKM values of *CUL3* are similar to FPKM values of S7, so it would be expected that EM1 should have produced a Ct value. Although the experiment was repeated, it was possible that the EM1 sample did not work, due to the lack of a template that Taqman primers required for hybridisation. From the FPKM values, it was expected that samples extracted from Seramir and MiRCURY would express higher than samples from ExoRNasy and EM methods. However, there was very little difference in the expression between extraction methods.



FPKM values	S2	S7	M7	M8	E7	E8	EM1	EM7	EM8
	54.54	34.15	37.29	72.17	33.36	26.98	28.47	1.67	22.36

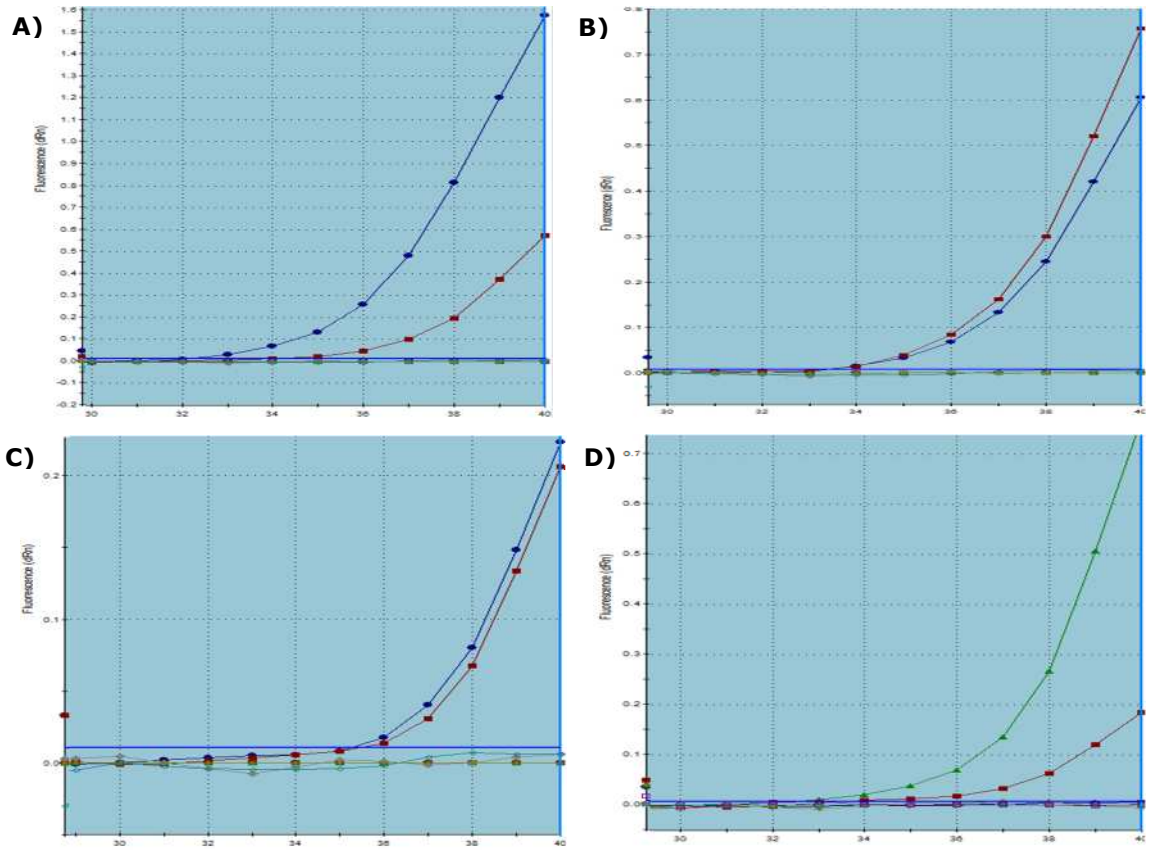
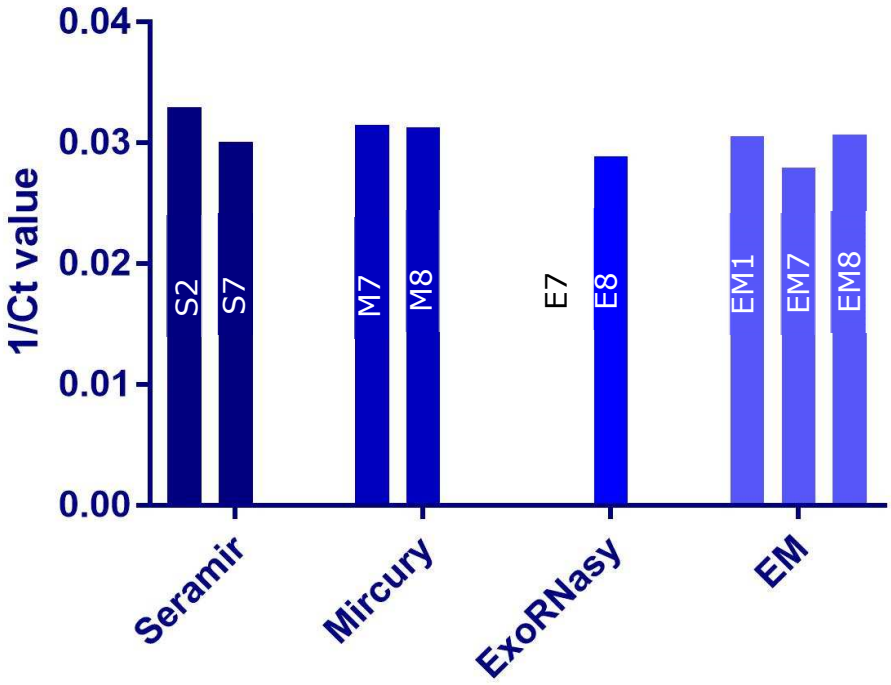


Figure 5-7 qRT-PCR Ct values of CUL3 expression. *Each sample sequenced were validated using qRT-PCR and compared to each other. The table below the bar chart shows the FPKM values obtained using RNA sequencing from the same samples. Amplification plots show amplification of each of the 9 samples. A=Samples S2 and S7. B=Samples M7 and M8. C=Samples E7 and E8. D=Samples EM1, EM7 and EM8.*

5.2.6.2 WNK1

As with *CUL3*, FPKM values and qPCR values do not correlate each other. Those with higher FPKM values e.g. E7 have no Ct value, but E8 with a lower FPKM value, produced a Ct value. As with CUL3 qPCR results, a lack of a Ct value for E7 could be explained by the absence of the specific template the CUL3 primers required for hybridisation.

The same can be said for SeraMir samples, the higher Ct values (S2) do not represent the low FPKM values seen from RNA-seq (S2=14.84). However, overall qRT-PCR results showed SeraMir and MiRCURY as the extraction methods, which appear to give the highest levels of expression.



FPKM values	S2	S7	M7	M8	E7	E8	EM1	EM7	EM8
	14.84	39.94	29.34	16.60	15.09	9.61	19.23	48.14	19.68

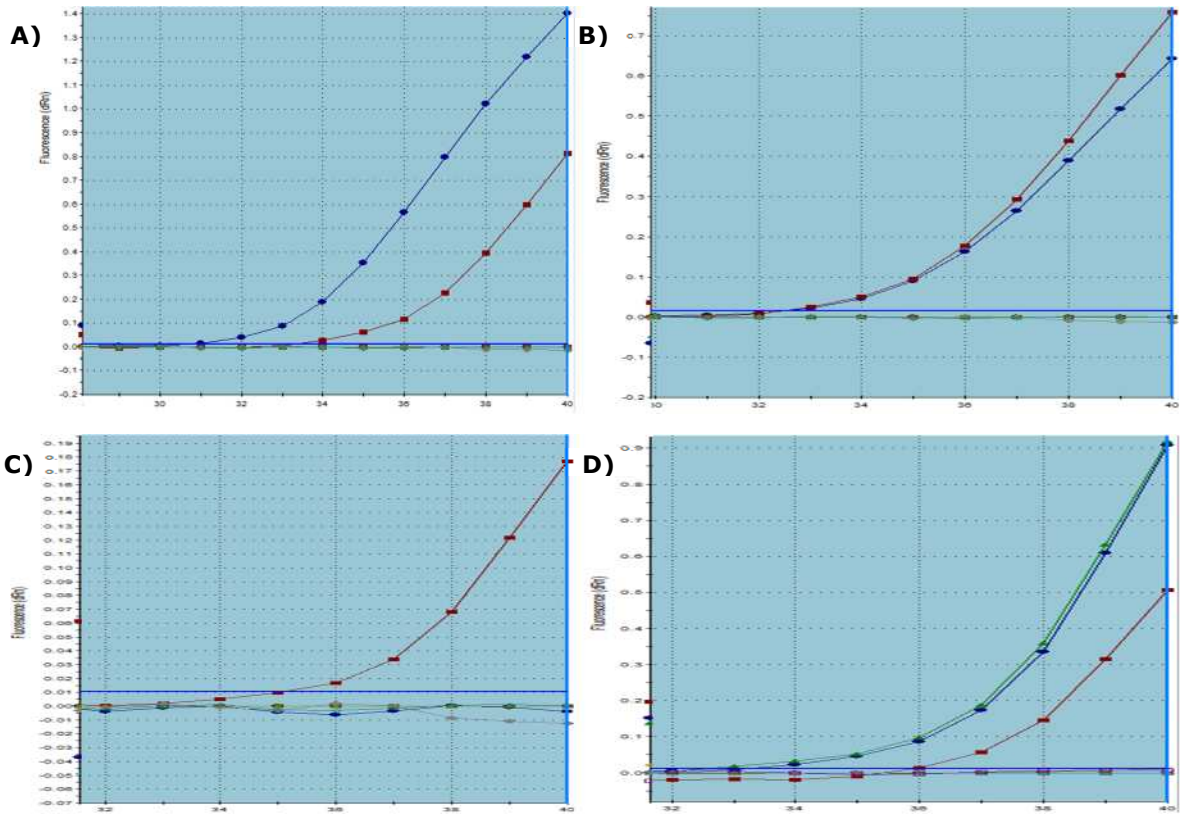


Figure 5-8 qRT-PCR Ct values of WNK1 expression. *As with CUL3, each sample seen above were validated against RNA-sequencing. The table below the bar chart describes each sample's associated FPKM values. A=Samples S2 and S7. B=Samples M7 and M8. C=Samples E7 and E8. D=Samples EM1, EM7 and EM8.*

5.2.6.3 Q-PCR comparison across extraction methods

Figure 5-9 compares all 3 genes (*WNK1, CUL3* and *RACK1*) from the 4 top extraction methods and their 1/Ct values. As *RACK1* presented with high FPKM values, it was used to compare amplification, against *CUL3* and *WNK1*, but for not for normalisation due to the high Ct values produced.

In qPCR, *CUL3* was expressed in all 4 methods, but the same was not seen with *WNK1*. The absence of *WNK1* has been explained above. Although RNA-sequencing measures the expression by the final amount of RNA and qRT-PCR measures expression by amplification, both methods showed there was little difference in Ct values and FPKM values between extraction methods.

By using results from the validation stage, RNA-sequencing quality controls and qRT-PCR results can be concluded that urinary exosomal RNA can be successfully extracted and sequenced, using SeraMir.

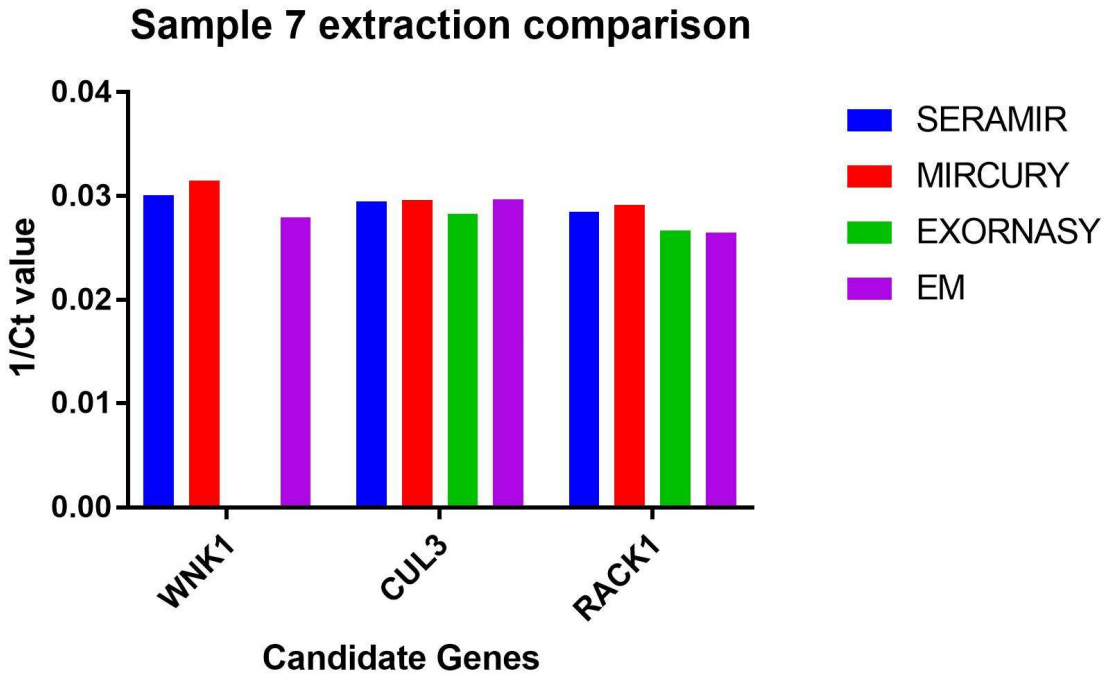


Figure 5-9 Comparison of Sample 7 across the top 4 extraction methods. *From the qRT-PCR results, MiRCURY produced the highest 1/Ct value, although there was very little difference between all of the top 4 extraction methods. As with Figure 5-8, the RNA extracted by ExoRNAsy did not produce at Ct value, and therefore absent from the graph.*

5.3 Discussion

The aim of the work described in this chapter was to compare published exosomal mRNA extraction methods and identify the best ones to take forward for RNA-sequencing studies using healthy volunteer samples. This chapter has shown that urinary exosomal mRNA can be extracted to a standard suitable for RNA-sequencing but that the yields and quality are very poor.

5.3.1 Extraction methods

Overall, Seramir produced the best results for extracting urinary exosomal mRNA. Seramir samples contained the highest quality and quantity mRNA extracted and showed higher FPKM values from RNA sequencing, which was confirmed by validation qRT-PCR experiments for *WNK1* and *CUL3*. Tang *et* al. also showed Seramir in combination with an exosomal extraction was a feasible method for the best RNA recovery [180].

Noticeably, there was some variability observed between all 11 extraction methods, despite the same samples used. Tang *et* al. also presented this observation, therefore highlighting the importance of using the same extraction methods to compare between samples [180]. However, FPKM values varied very little between the extraction methods selected from RNA-sequencing.

Seramir and MiRCURY methods were better at extracting mRNA in comparison to other extraction methods. FPKM values of *PDCD6IP* (ALIX) were higher using the Seramir extraction method, but no values were seen using ExoRNasy extraction method. This observation suggests that some extraction methods cause degradation of the *PDCD6IP* (ALIX) mRNA, as all urinary samples were collected and processed at the same time. This is also similar for *WNK2* where its mRNA is only expressed in samples extracted with Seramir. It may be suggested that different RNA extraction methods should be adopted depending on the RNA genes/species to be extracted although there is no known way of predicting which RNAs will extract best with each method.

Ultrafiltration is the major exosomal isolation method used in this thesis. Whilst Exoquick and MiRCURY exosomal reagents were not successful in extracting high-quality mRNA, Exoeasy reagent accompanied with RNA extraction produced mRNA suitable for RNA-sequencing. However, many studies have shown that exosomal reagents target non-exosomal substances e.g. other EVs or RNA-binding proteins [179, 186, 187]. This could explain the variability seen in samples extracted with exosomal-binding reagents. Results in samples extracted by exosomal-binding reagents would be uncertain, as it is impossible to confirm if mRNA has come from exosomes. Despite showing similar qPCR gene expression to exosomes extracted by ultrafiltration, exosomal reagents are extremely expensive to use when extracting large quantities of samples, therefore making ultrafiltration a more appealing option.

This chapter and thesis focus mainly on extracting exosomes using ultrafiltration or exosomal reagents. However, ultracentrifugation is commonly used exosome purification method and has the potential to produce better mRNA quality and quantity due to its ability to separate the exosomes into a pellet. However, ultracentrifugation requires a centrifuge, which is capable of reaching above 100,000g. This resource was unavailable locally at the time of this work.

Although many studies use ultracentrifugation, this method of extraction does not always produce a pure exosome fraction like filtration does [102]. Opti-prep density gradient centrifugation is used in addition to standard ultracentrifugation, which produces a significantly higher quantity enriched exosome sample [132]. Van Deun *et* al. reported the RNA concentrations were of low quantity, but of those concentrations, this version of centrifugation generated 30% of all mRNA [132]. According to Van Deun, using the ultrafiltration method yields 6% of the total mRNA [132].

Whilst these methods have been well established, new nano-technology based methods may provide a better way to fully characterise exosomes, as well as quantify other extracellular vesicles [188]. Nano-technology could improve the identification of exosomes and RNA yield by using the Nanosight

LM10 instrument [188]. Huang et al. showed RNA concentrations of 10-15ng from the Agilent Bioanalyzer by using Nanosight LM10 technology [187]. This is a roughly a 4-fold increase on the concentrations achieved using Seramir RNA extraction in this chapter.

To improve mRNA quality for future studies, the addition of RNase inhibitor to the exosomal fraction could be used, to avoid as much mRNA degradation as possible. Preferably, RNase inhibitor should be also included upon urine collection, but as urine is usually collected in a clinical environment, this may be challenging, and the RNase inhibitor is also costly.

5.3.2 RNA-sequencing and qPCR

RNA-sequencing was able to detect a high range of genes from the urinary exosome samples, despite the limitation of RNA concentration and quality. The technique allowed for a broad reading of the whole genome, significantly more sensitive than qRT-PCR, detecting expression levels that would be under-represented if only using qRT-PCR.

Although *WNK1* and *CUL3* genes were detected with RNA-sequencing analysis, the qRT-PCR Ct value results did not reflect the results of the FPKM values. One explanation for this could be the fragments extracted from exosomes were too short for qRT-PCR experiments. TaqMan primers require certain length fragments for the PCR to show the true expression of genes, which if missing, results would suggest a lack of expression [189]. For example, RNA sequencing show fragment sequences of 52bp (Table 5-5), however, the *WNK1* primers require an amplicon length of 65bp or more. This explanation could also explain why *RACK1* did not produce a lower Ct value as expected.

Another explanation for the higher than expected Ct values for *RACK1* could be due to the location of the primers. *RACK1* primers were designed to bind to exons 1-2 (Thermofisher; Hs00272002_m1). Degradation of the sequence of the gene is more likely, due to its location at the ends of genes, therefore explaining why *RACK1* did not show high expression values as suggested from RNA-sequencing data. Primer sequences for *WNK1* and *CUL3* were located throughout the whole of the gene.

It can be concluded that RNA-sequencing is a more sensitive method of quantifying gene expression than qRT-PCR as it is not reliant on a specific sequence being still present in a sample. This feature of RNA-sequencing is particularly vital when working with urinary exosomal mRNA, due to natural degradation.

Although *AQP2*/*PDCD6IP* (ALIX) was shown as highly abundant as an exosomal protein, the gene expression FPKM values were too low to be considered for use as a house-keeper gene to normalise samples. Another study found extracting samples with the ultrafiltration method also showed low *AQP2* and *PDCD6IP* (ALIX) expression, whilst exosomes extracted through ultracentrifugation showed a high level of *AQP2* and *PDCD6IP* (ALIX) expression [174]. This could suggest that the results from this chapter maybe have been affected by the extraction of the exosomes. It is also possible that the specific protein is more stable than others, hence less mRNA is required to maintain protein levels. Usually, *β-actin* and *GAPDH* are used as house-keeper genes for qRT-PCR, however, their associated FPKM values were also not expressed high enough to be considered.

The chapter has shown that urinary exosomal mRNA can be extracted and analysed using RNA-sequencing. Considering all quality control methods, RNA-sequencing FPKM values and qRT-PCR expression results, it can be concluded that SeraMir was the best exosomal mRNA extraction method, followed by MiRCURY. However, overall the quality of RNA extracted, and the yields were lower than that seen from samples that are more traditional e.g. extracted directly from cells in culture or from tissue biopsies. Whilst *WNK1* and *CUL3* were detected in exosomes, *SLC12A3* (NCC) and other related proteins were not expressed. However, by adding RNase inhibitor to future samples, this may reduce RNA degradation that may have limited the data observed here.

Chapter 6: Differential gene expression between acute and convalescent Thiazide-Induced Hyponatraemia patients

6 Differential gene expression between acute and convalescent Thiazide-Induced Hyponatraemia patients

Abstract

Thiazide diuretics, prescribed to control high blood pressure, can cause Thiazide Induced Hyponatraemia (TIH) in a minority of patients. The condition is caused by a reduced concentration of serum sodium, with symptoms ranging in severity depending on the concentration of sodium in the blood.

Recently, genetic studies have shown a predisposition to TIH, suggesting that some patients are more likely to develop the condition if they express certain genes. This has been confirmed with NCC and AQP2 protein studies in acute and convalescent TIH patients.

Using the SeraMir extraction method detailed in Chapter 5 and the addition of RNase inhibitor, urinary exosomal RNA was extracted from acute and convalescent TIH and control urine samples. The samples were then sent for RNA sequencing at Oxford Genomics, before analysing the data in-house. The aim of this project was to compare acute and convalescent TIH exosomal samples, identifying mRNA differing in expression between cases and controls, as well as comparing differential expression between acute and convalescent TIH cases.

Despite using both the Hypothesis Free Method and Candidate Method to analyse individual urinary exosomal samples, no known RNAs associated with TIH were identified. *SLC12A3* was identified as differential expressed between acute and convalescent TIH samples, but upon closer analysis, it was concluded that this differential expression was driven by one sample, and therefore excluded from further analysis.

This chapter identified *GYS2* and *SLC5A2*, which may suggest a possible link to the glucose and sodium relationship within the condition. Further pathway analysis, as well as genomic and proteomic analysis would help identify with these glucose-related proteins affect the pathophysiology of TIH patients.

6.1 Introduction

Thiazide Hyponatraemia (TIH) is a condition caused by thiazide diuretics, drugs taken by patients with hypertension. A reduced serum sodium concentration is observed as a side effect in a minority of patients. The severity of symptoms depends on acuity and sodium concentration. Moderate TIH has a sodium concentration of 125-130mM, whilst the most severe TIH patients have a serum sodium concentration of less than 125mM [190]. These patients require hospitalisation and immediate withdrawal of thiazide diuretics [190].

The mechanism of action of thiazide and thiazide-like diuretics is the same, involving binding to the thiazide-sensitive sodium-chloride cotransporter (NCC) [3]. Intolerance to the drug can occur either within hours or over years of taking the drug [39].

It has been suggested that there may be genetic predisposing factors to explain a predisposition to the development of TIH. In support of this hypothesis is the observation that thiazides do not significantly change the sodium concentration within the blood in the total thiazide treated population, implying there must be a proportion of the population that have an increased susceptibility towards developing TIH [81].

In a pilot genome-wide association study (GWAS) of 48 hospitalised patients, SLCO2A1 (prostaglandin transporter (PGT)) was identified as significantly associated with the development of TIH, and therefore studied further [77]. Sequencing of the SLCO2A1 showed that some patients had a variant (pA396T), which in *in vitro* studies was shown to reduce the activity of the PGT protein [77]. However, not all acute TIH patients expressed the pA396T, suggesting other mechanisms must also exist to cause the condition.

NCC and AQP2 exosomal protein expression show a significant difference between acute and convalescent TIH patients and also with normonatraemic controls [117]. Figure 6-1 shows the protein expression of AQP2 (A), NCC_3 and $NCC_{1/2}$ between acute, convalescent and control patients.

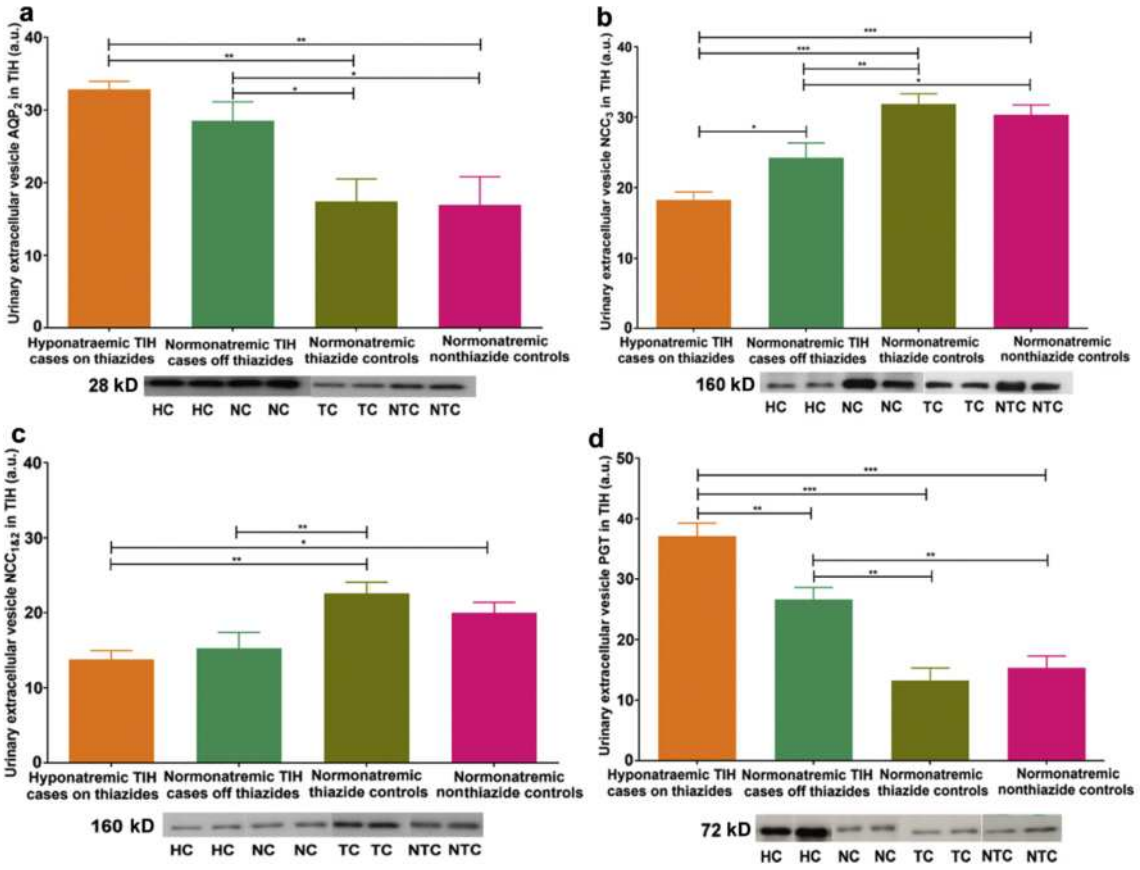


Figure 6-1 AQP2, NCC and PGT protein expression in TIH patients and controls. *HC = Hyponatraemic TIH cases on thiazides; NC = Normonatraemic TIH cases off thiazides; TC = Normonatraemic Thiazide controls; NTC = Normonatraemic non-thiazide controls. A) AQP2 is expressed significantly higher in acute and convalescent TIH patients than controls. B) NCC_3 is highly expressed in controls in comparison to acute and convalescent TIH patients. It is also significantly different between acute and convalescent. C) $NCC_{1/2}$ shows no significant expression difference between acute and convalescent patients but is significantly different against controls. D) Acute and convalescent TIH patients' expression PGT significantly higher than controls. Reproduced with permission [117].*

Whilst Figure 6-1 shows that NCC_3 and $NCC_{1/2}$ expression was lowest in those with acute TIH when compared to convalescent and controls, Channavajjhala *et* al. described a significant increase in phosphorylation of 3 N-terminal sites of NCC (T55, T60 and S91) in acute TIH [117]. Expression of these three phosphorylated sites was significantly reduced in convalescent and controls, suggesting that in patients with acute TIH, there is an increase in phosphorylated NCC [117]. These results of increased phosphorylated NCC and reduced NCC_3 in acute TIH patients could suggest NCC_3 activation by phosphorylation is a compensatory response to reduced NCC expression.

In addition to AQP2 and NCC, *KCNJ1* (ROMK) has also shown a potential association for the development of TIH in some patients [191]. ROMK is highly expressed in the TAL and CD, which causes sodium wastage with a loss-of-function phenotypic mice ($ROMK^{-/-}$) [192, 193]. A genomic study found statistical associations with acute TIH patients and KCNJ1 rs2509585 C/T or T/T polymorphisms [191].

Using the SeraMir exosomal RNA extraction kit detailed in the previous chapter, TIH patient urinary exosomal samples were extracted and outsourced to Oxford Genomics for RNA-sequencing. Data analysis was performed in-house, after the raw data files had been received from Oxford Genomics. In order to attempt to improve RNA quantity and quality, RNase inhibitor was added before exosomal isolation and before exosomal RNA extraction. The aim of this project was to compare acute and convalescent patients, identifying mRNA differing in expression between cases and controls, as well as comparing differential expression between acute and convalescent TIH cases.

6.2 Results

As with chapter 5, I performed all exosomal and RNA extraction. Before RNA samples were sent to Oxford Genomics for RNA sequencing, I also measured the RNA quantity and quality by Ribogreen assays (see Section 2.6.3). Once RNA sequencing was complete, Oxford Genomics sent make the raw fastq data files generated by the HiSeq4500 machine. All data analysis was performed in-house by myself.

In total, 40 spot urine samples were selected for exosomal and RNA extraction and sequencing. Of these 40 patients, 10 samples were from patients with acute TIH (collected after hospitalisation), 10 samples were convalescent TIH patients (collected once recovered and normonatraemic) and 20 were control samples (10 normonatraemic thiazide controls (C1) and 10 normonatraemic off thiazide controls (C2)). Acute TIH samples will be compared to C1 control samples, whilst Convalescent TIH samples will be compared against C2 control samples.

Acute samples were collected upon admission to hospital; whilst convalescent samples were collected two months later in the outpatient department (once off thiazide diuretics).

6.2.1 RiboGreen validation

The RiboGreen method used in Chapter 5 was the quality control method performed in this chapter to measure the concentration of mRNA, as using the Nanodrop produced false positive results in Chapter 5. Quantifying mRNA using the Bioanalyzer was not performed in this chapter to avoid duplication of results, as Oxford Genomics used the Bioanalyzer to measure mRNA concentration.

Overall, mRNA concentrations were low as expected with exosomal mRNA. Acute, convalescent and control groups had similar concentrations (11.23±6.38, 11.49±9.14, and 11.45±8.74, respectively). The total mRNA shows the concentration of mRNA in 25mL of urine. This also did not vary differently between acute, convalescent and control groups (561.43±319.05, 574.42±457.19, and 572.33±437.04, respectively).

These concentrations were similar to those seen in the work described in chapter 5. As samples were low in concentration, they were concentrated by AMPure XP beads (by Oxford Genomics), before continuing to RNA-sequencing.

Table 6-1 Ribogreen results showing mRNA concentration of TIH and GS samples. *RNA concentrations have been calculated using the Ribogreen assay per ng/µL, Total RNA (in 25mL of urine) and ng/mL of urine. Concentrations have been averaged according to the severity of TIH and controls.*

Samples	Conc (ng/µl)	Total RNA	ng/ml Urine	Samples	Conc (ng/µl)	Total RNA	ng/ml Urine	Samples	Conc (ng/µl)	Total RNA	ng/ml Urine
Acute1	5.38	268.95	10.76	**Conv1**	13.99	699.25	27.97	**Cont1**	28.73	1436.50	57.46
Acute2	22.31	1115.5	44.62	**Conv2**	6.75	337.25	13.49	**Cont2**	19.51	975.40	39.02
Acute3	17.86	892.75	63.71	**Conv3**	29.88	1494.1	59.76	**Cont3**	2.65	132.70	5.31
Acute4	3.54	176.90	7.08	**Conv4**	5.61	280.55	11.22	**Cont4**	2.85	142.35	5.69
Acute5	10.17	508.35	20.33	**Conv5**	2.76	137.95	5.12	**Cont5**	3.01	150.35	6.01
Acute6	9.54	476.90	19.08	**Conv6**	9.15	457.28	18.29	**Cont6**	7.61	380.30	15.21
Acute7	11.43	571.60	22.86	**Conv7**	14.40	720.04	28.80	**Cont7**	18.06	902.75	36.11
Acute8	11.42	570.9	22.84	**Conv8**	4.96	248.09	9.92	**Cont8**	15.61	780.50	31.22
Acute9	17.42	871.00	34.84	**Conv9**	23.90	1195.2	47.81	**Cont9**	24.62	1230.90	49.24
Acute10	3.23	161.45	6.46	**Conv10**	3.49	174.50	6.99	**Cont10**	16.75	837.35	33.50
								Cont11	6.45	322.70	12.91
Average	11.23	561.43	22.46	**Average**	11.49	574.42	22.98	**Cont12**	4.57	228.70	9.15
S.D	6.38	319.05	12.76	**S.D**	9.14	457.19	18.29	**Cont13**	3.42	170.85	6.83
								Cont14	1.64	81.85	3.27
								Cont15	23.94	1197.15	47.89
								Cont16	3.37	168.50	6.74
								Cont17	17.66	883.15	35.32
								Cont18	16.72	836	33.44
								Cont19	7.55	377.35	15.09
								Cont20	4.22	211.20	8.45
								Average	11.45	572.33	22.89
								S.D	8.74	437.04	17.48

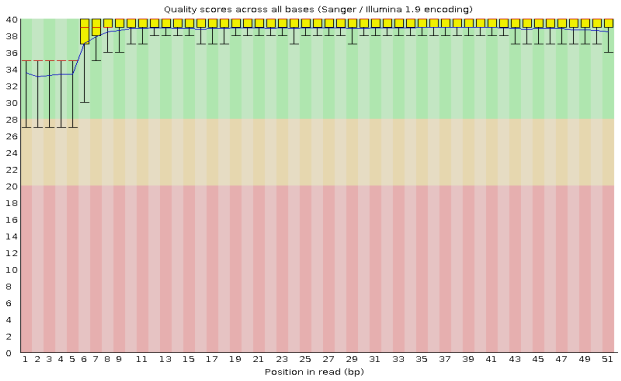
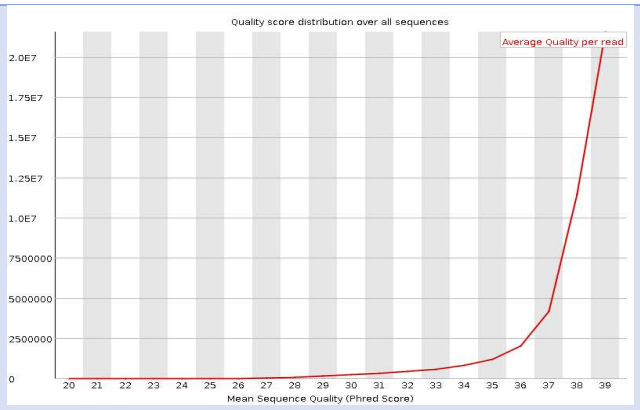
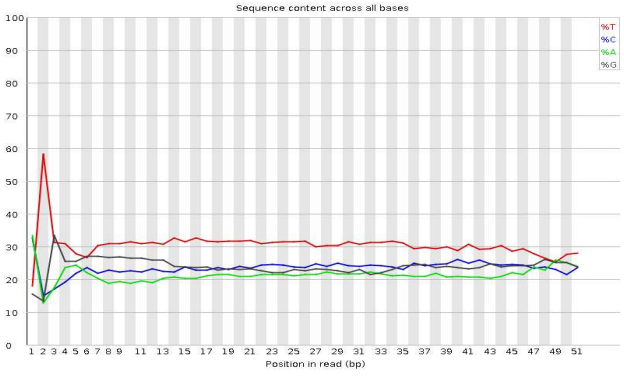
6.2.2 RNA-sequencing

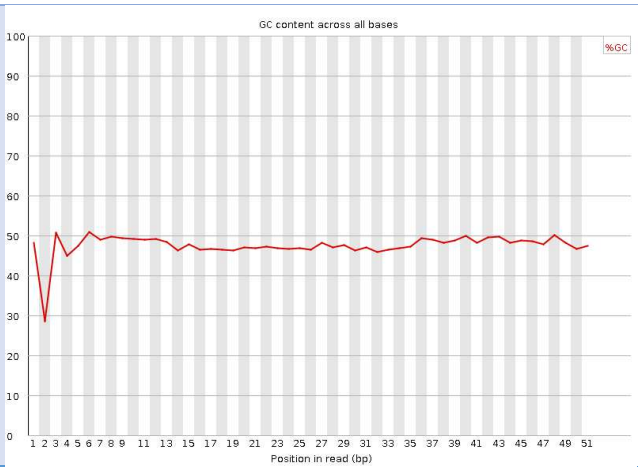
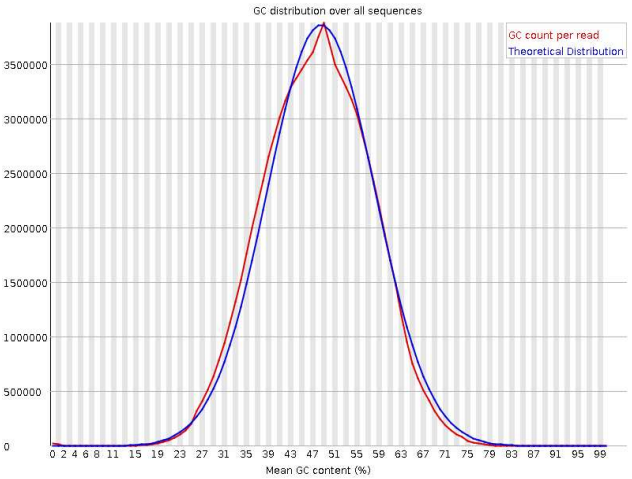
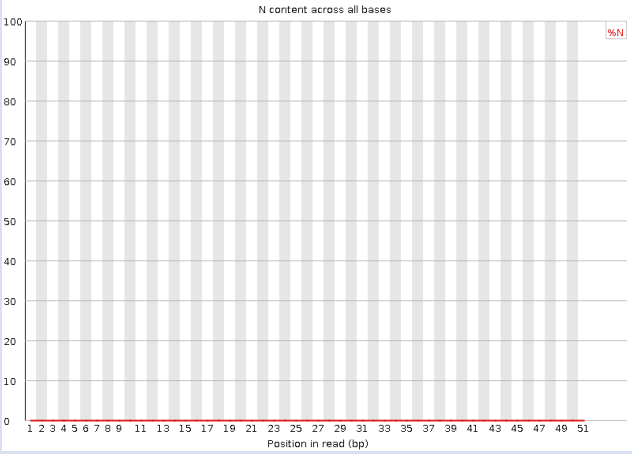
Due to the nature of working with exosomal mRNA, the library quality (i.e. from sample preparation for RNA-sequencing and cDNA synthesis) was low in concentration, as expected from Chapter 5. Samples had been concentrated and amplified to increase the chances of a higher read count. However, despite amplification and concentrating, library preparation still produced RNA concentrations too low to use the HiSeq4500. As the samples were concentrated, the material was lost, so the decision was made to run one pool (every sample on one flowcell) to examine gene expression. The majority of these samples provided a good quality control check, high percentage of alignment and FPKM values. However, 6 control samples were too weak to continue with further re-pooling in RNA-sequencing and removed from further analysis. This resulted in 6x C1 control samples and 8x C2 control samples. As Section 2.8.2 describes, the remaining 34 samples (10 acute, 10 convalescent and 14 control) were re-pooled by volume in order to achieve the target of 30 million reads per sample. The following sequencing results are of the 34 successful samples from the initial pool.

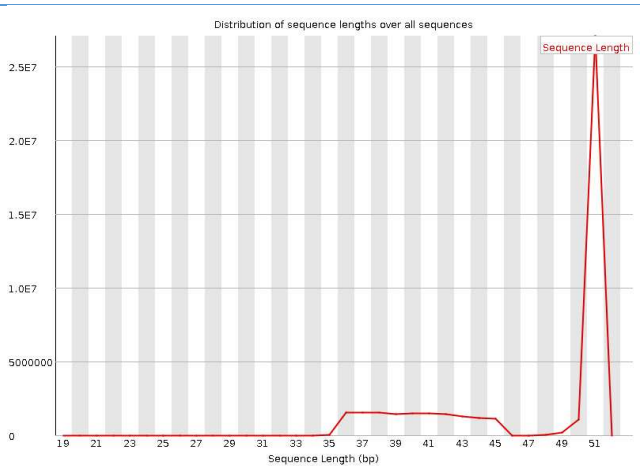
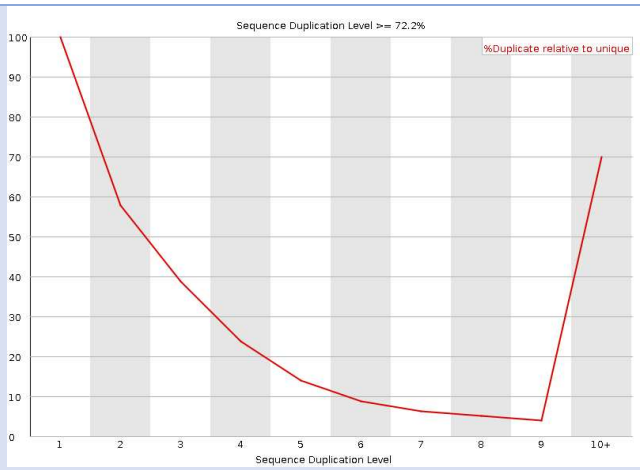
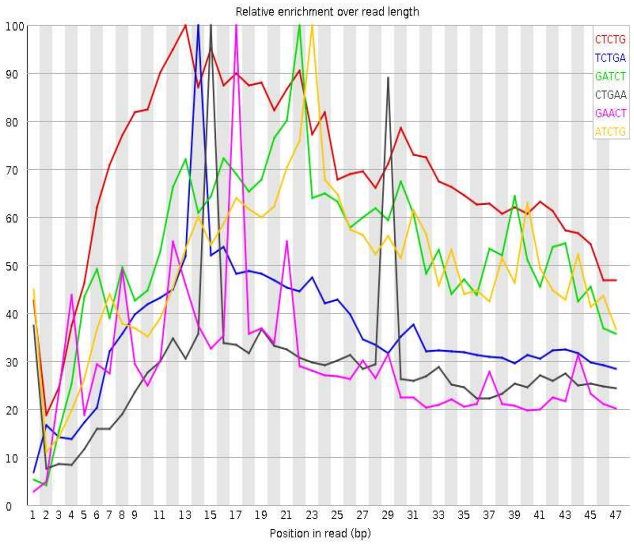
6.2.3 Sequencing quality control

As with Section 5.2.2, all RNA sequencing analysis must include a Fastqc Quality Control. All samples sequenced passed quality control checks. Table 6-2 shows an example of the results generated from the command "fastqc":

Table 6-2 Acute 1 Fastqc report. *Graphs and data shown were used to decide the continuation of RNA-sequencing. Graphs of most importance are "per base sequence quality", "Per Base Sequence Content", "Per Base GC Content", and "Per Sequence GC Content". All other samples showed similar results.*

FastQC module	Function
Basic Stats	Total Sequences: 43416318 Filtered Sequences: 0 Sequence Length: 20-51 %GC: 47
Per Base Sequence Quality	
Per Sequence Quality Scores	
Per Base Sequence Content	

Per Base GC Content	
Per Sequence GC Content	
Per Base N Content	

Sequence Length Distribution	
Duplicate Sequences	
Overrepresented Kmers	

6.2.4 Alignment against the Human Genome

The TopHat programme aligns samples against the Human Genome. The command line produces results showing the alignment of each sample as a percentage. In this thesis, samples were aligned to the Human Genome version 37.

Alignments between acute, convalescent and control groups were high, considering the low concentrations of mRNA. There was very little difference between the alignment percentages between all three groups (acute =67.42%±0.25, convalescent=60.71%±0.17 and controls=68.69%±0.19).

Read depth was also another important factor to quality control checks, as it shows a deeper coverage has been read of each sequence. This deeper coverage means there is a higher level of confidence that the sequence alignments made are true.

All samples produced more reads than expected (30 million reads per sample), allowing confidence in the results produced by Cuffmerge and CuffDiff. All groups were similar in read depth (acute=$2.72x10^8$, convalescent=$2.71x10^8$ and controls=$2.76x10^8$).

The alignment percentage and the read depth, along with the FastQC show that the samples were therefore considered acceptable to continue with RNA-sequencing analysis.

Table 6-3 Alignment percentage and Read Depth of all samples sequenced. *The alignment percentage shows how much of each sample has aligned with the Human Genome version 37. The read depth shows how many times the sample was read by the HiSeq2500 machine.*

Samples	Alignment %	Read depth	Samples	Alignment %	Read depth	Samples	Alignment %	Read depth
Acute1	79.4%	$2.51x10^8$	**Conv1**	61.6%	2.96E+08	**Control G1-1**	77.0%	3.15E+08
Acute2	83.7%	$3.18x10^8$	**Conv2**	49.8%	2.65E+08	**Control G1-2**	49.1%	2.54E+08
Acute3	10.4%	$2.55x10^8$	**Conv3**	85.5%	2.86E+08	**Control G1-3**	54.2%	2.55E+08
Acute4	37.8%	$2.42x10^8$	**Conv4**	67.9%	2.66E+08	**Control G1-4**	83.2%	2.89E+08
Acute5	84.2%	$2.53x10^8$	**Conv5**	67.4%	2.46E+08	**Control G1-5**	74.7%	2.84E+08
Acute6	77.1%	$2.40x10^8$	**Conv6**	65.6%	2.80E+08	**Control G1-6**	19.1%	2.80E+08
Acute7	84.8%	$2.82x10^8$	**Conv7**	41.7%	2.40E+08	**Control G2-1**	83.8%	2.68E+08
Acute8	80.1%	$2.99x10^8$	**Conv8**	47.6%	2.95E+08	**Control G2-2**	63.8%	2.46E+08
Acute9	83.0%	$2.90x10^8$	**Conv9**	83.3%	2.90E+08	**Control G2-3**	82.8%	2.98E+08
Acute10	53.7%	$2.83x10^8$	**Conv10**	36.7%	2.51E+08	**Control G2-4**	76.2%	3.15E+08
						Control G2-5	80.1%	2.92E+08
Average	67.42%	$2.71x10^8$	**Average**	60.71%	$2.72x10^8$	**Control G2-6**	83.2%	2.90E+08
S.D	0.25	$2.67x10^7$	**S.D**	0.17	$2.08x10^7$	**Control G2-7**	63.8%	2.39E+08
						Control G2-8	70.6%	2.43E+08
						Average	68.69%	$2.76x10^8$
						S.D	0.19	$2.57x10^7$

6.2.5 Differential expression of mRNA between Acute and Convalescent TIH patients

Two approaches were taken to analysis the data below. Firstly, a hypothesis-free method was carried out, using directional fold change (log2(fold_change)) and fold change (calculated from log2(foldchange)) to identify the top differentially expressed mRNA.

The second part of the analysis was to use a candidate mRNA approach, which identifies those genes/proteins implicated in TIH pathology.

To compare gene expression between acute TIH, convalescent TIH, and control patient samples, the Cuffdiff tool was used as the last part of the RNA-sequencing analysis. As Section 2.8.3.4, Cuffdiff compares two different conditions, producing results with statistical significance. The results below were presented based on either their fold change (False Discovery Rate) or their q value (corrected p-value, reducing the chance of false positives). Comparison groups were:

- Acute TIH samples vs Convalescent TIH samples
- Acute TIH samples vs controls (C1 - normonatraemic on thiazide)
- Convalescent TIH samples vs controls (C2 - normonatraemic off thiazide)

6.2.5.1 Differential expression with Fold Change

Figure 6-2 shows a Venn diagram of the top 20 genes expressed determined by fold change. The convalescent vs C2 controls and acute vs convalescent have the most genes in common (3 differential expressed genes). These genes included FYVE, RhoGEF And PH Domain Containing 1 (*FGD1*), *C9orf57* (Uncharacterised protein) and *RP11-429P3.5* (a pseudogene). Acute vs C1 controls had the most genes different from the rest of the group. These included Small Nucleolar RNA Host Gene 6 (*SNHG6*), Carboxypeptidase A6 (*CPA6*), FAT Atypical Cadherin 3 (*FAT3*) and Glutamic-Oxaloacetic Transaminase 1 *(GOT1)*. *GOT1* is a phosphate-dependent enzyme found in the mitochondria and the cytoplasm, whilst *CPA6* is involved in the production of amino acids. No transporter protein mRNA's are found in the top 20.

Figure 6-3 shows a Venn diagram of all differential expressed genes between the three comparison groups. Only 22 genes were differentially expressed between all three groups, these including protein-coding mRNA's and some non-coding mRNA's. Only 5 genes are similar between Convalescent vs C2 controls and acute vs C1 controls (*CCDC3, RP3-468B3.2, DOCK4, DUX4L17,* and *GPR126*), whilst 264 genes were similar between acute vs C1 convalescent and convalescent vs C2 controls.

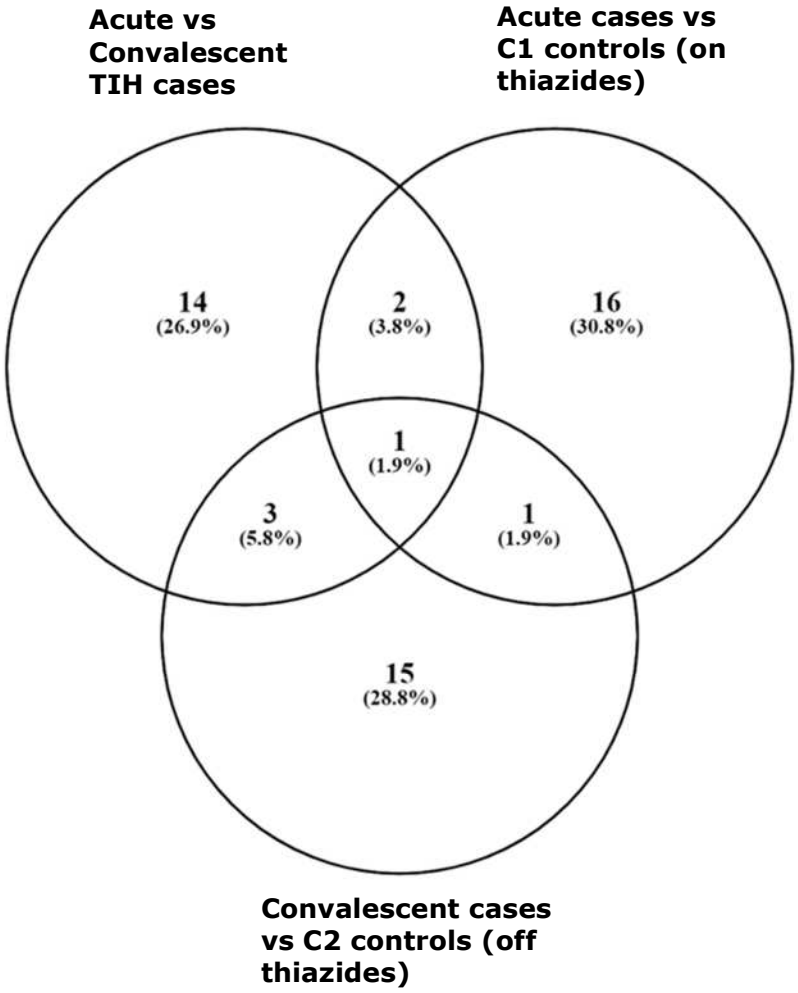


Figure 6-2 Top 20 gene comparisons between Acute, convalescent and controls by fold change. *The Venn diagram shows the similar genes between groups. There was 1 identical gene differentially expressed between all three comparisons (SNGH6).*

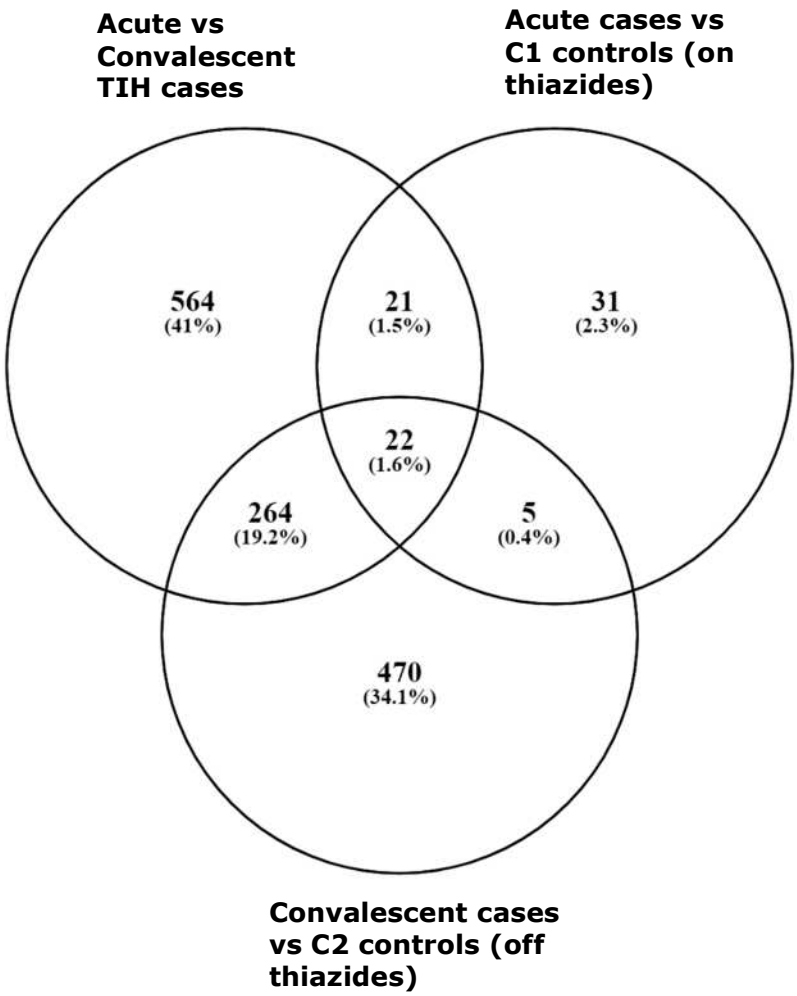


Figure 6-3 All differential expressed genes compared against Acute, Convalescent and control groups by the fold change. *The Venn diagram shows the genes that are similar and different between the 3 comparison groups.*

6.2.5.1.1 Acute TIH vs Convalescent TIH

Table 6-4 shows the top 20 genes between Acute TIH samples and convalescent TIH patient samples when ordered by fold change. Most of the genes are pseudogenes or non-coding genes e.g. *RP11-432J9.6* or small nucleolar RNA (*SNHG6*). Some of the genes in this top 20 are involved in cell regulation e.g. RNA polymerase subunit (*POLR2*), DNA polymerase interacting subunit (*POLDIP2*). The only transport protein was SLC35F1, the function of which is still unknown, however, a recent study has shown high expression in the brain and kidney [194].

Table 6-4 Top 20 genes expressed between Acute TIH samples and Convalescent TIH when ordered by fold change. *The top 20 genes are listed by fold change. The log2 (fold change) is also listed to show direction.*

Gene name	Acute FPKM	Convalescent FPKM	log2(fold_change)	Fold change (2^_)	q-value
SNHG6	0.17	1366.28	12.94	7845.23	0.000755
ADAM20	1082.30	0.33	-11.67	3262.13	0.000755
POLR2J	595.39	0.36	-10.71	1672.40	0.014555
CPA6	373.42	0.26	-10.47	1420.02	0.010929
FGD1	0.64	794.99	10.27	1236.97	0.002626
AC004231.2	868.95	0.71	-10.27	1230.05	0.019585
LINC00452	358.93	0.30	-10.22	1191.20	0.021916
RP11-432J9.6	610.06	0.58	-10.05	1059.23	0.022598
C9orf57	1.81	1869.59	10.01	1034.20	0.002078
MTATP6P3	0.02	11.92	9.66	810.15	0.000428
SLC35F1	0.95	674.83	9.48	712.76	0.001582
RP11-626P14.2	1.33	702.48	9.05	528.42	0.000428
GYS2	0.88	458.65	9.03	523.92	0.000428
PTCH2	1.20	552.61	8.85	461.87	0.000428
RP11-867G23.8	4.47	1318.43	8.20	294.97	0.040698
RP11-6B6.3	5.23	1472.19	8.14	281.35	0.006541
PCDH9	253.31	0.92	-8.10	275.09	0.000428
POLDIP2	244.88	0.89	-8.10	274.72	0.003341
EPHA7	306.43	1.13	-8.08	271.37	0.001072
RP11-429P3.5	455.04	1.91	-7.90	238.71	0.001072

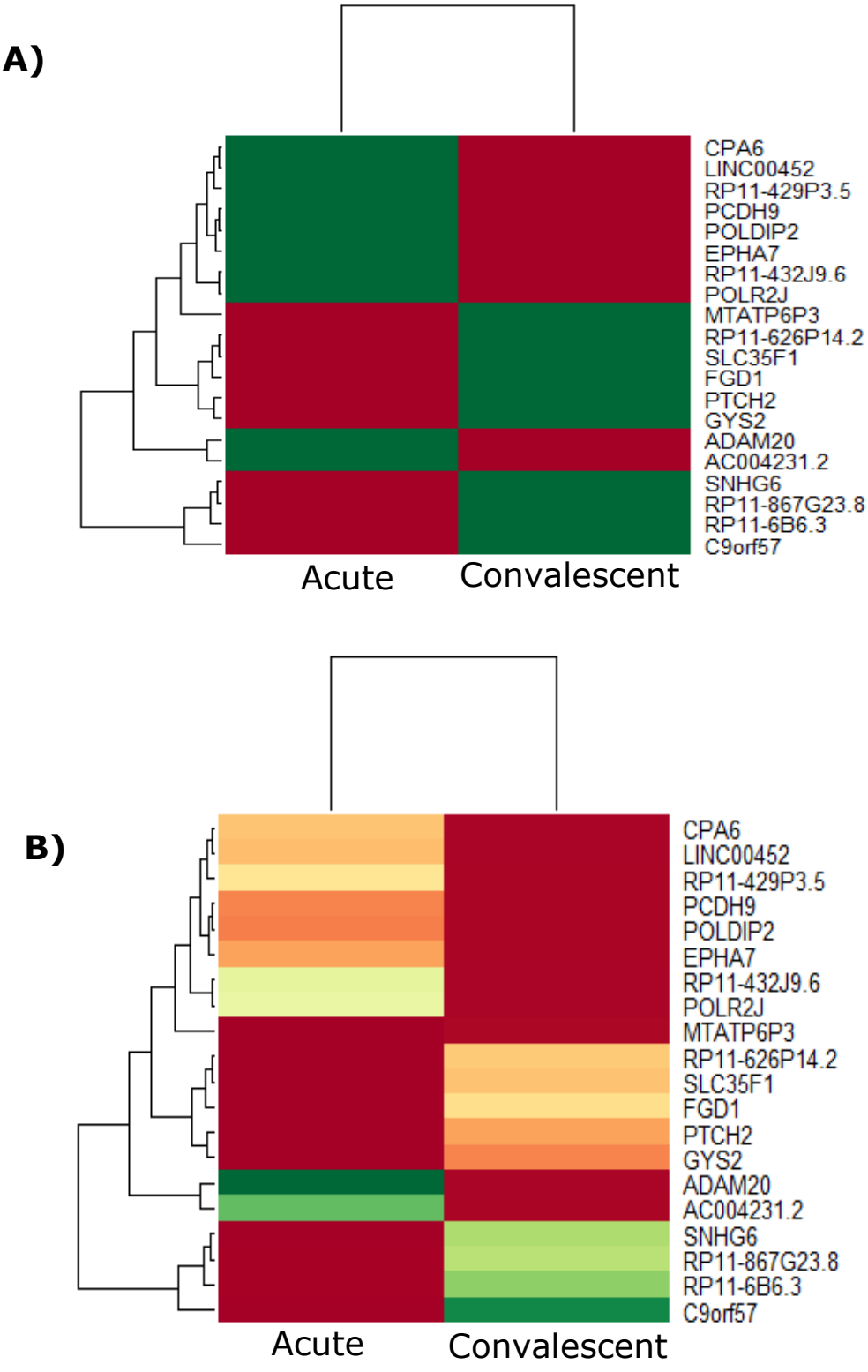


Figure 6-4 Heatmap of the top 20 differential expression genes between acute and convalescent TIH samples. *Red =lowly expressed mRNA, Cream = mid-expression mRNA Green = high expressed mRNA. A) The heatmap shows the standard differential expression, as seen in the table. B) This heatmap shows differential expression once the columns have been normalised within the column/population.*

Heatmaps have been used to visually represent differential gene expression. Each column represents a different condition (acute or convalescent or controls), whilst each row represents a different gene. Heatmaps can be used to visually display dominantly expressed genes, or genes that are relatively expressed between conditions.

Figure 6-4 describes the top 20 genes differentially expressed between acute and convalescent TIH samples as a heatmap. Whilst the top panel of Figure 6-4 shows a visual display of the data in Table 6-4 between differentially expressed mRNAs of acute TIH samples and convalescent TIH samples, the bottom panel of Figure 6-4 has been normalised within each condition, showing the relative impact of changes of gene expression within the acute and convalescent TIH samples. The bottom panel of Figure 6-4 shows the expression of *ADAM20 and AC004231.2 are* very high in acute TIH samples when compared to convalescent TIH samples, where they are among the lowest expressed within the convalescent TIH samples.

SNHG6, RP11-867G23.8, RP11-6B6.3, and *C9orf57* were the lowest expressed RNA's in the convalescent TIH samples; however, these RNA's were amongst the highest expressed in acute TIH samples relative to the other genes within this population.

6.2.5.1.2 Acute TIH vs C1 controls

Table 6-5 lists the mRNA differentially expressed between Acute TIH samples and C1 control samples. The majority of the top 20 mRNA's are protein coding, e.g. Mediator Complex Subunit 27 (*MED27*), Glutamic-oxaloacetic transaminase (*GOT1*) and FAT Atypical Cadherin 3 (*FAT3*). These genes are involved in normal gene function, for example, *MED27* is part of a complex of other proteins involved in activating transcription of the DNA, whilst *GOT1* is involved in the production of amino acids and urea.

There was also an overlap with some genes from acute TIH vs convalescent TIH (Section 6.2.5.1.1), which was confirmed by the Venn diagram seen in Figure 6-2 e.g. *CCDC3*. This comparison group show no statistically significant differences in expression of any mRNAs encoding renal electrolyte transporters. With this differential comparison, only 109 significantly differentially expressed mRNA were listed between acute TIH samples and the C1 controls.

Table 6-5 Top 20 genes expressed between Acute TIH samples and C1 controls when ordered by fold change. *The top 20 differentially genes are listed by fold change. The log2 (fold change) is also listed to show direction.*

Gene name	Acute FPKM	C1 Control FPKM	log2(fold_change)	Fold change (2^_)	q-value
RNF39	0.78	9394.38	13.55	11975.51	0.0407
MED27	2.13	6076.53	11.48	2846.55	0.01994
CCDC3	0.30	626.20	11.05	2115.09	0.040156
EEF1A1	8.76	10648.00	10.25	1215.98	0.00707
C17orf64	2357.87	2.98	-9.63	791.72	0.015002
SNHG6	0.16	116.97	9.51	731.58	0.033331
DLEU2	0.61	311.45	9.01	514.64	0.028204
EPHA7	283.17	0.84	-8.39	336.14	0.042604
GOT1	3.08	1009.94	8.36	327.56	0.013527
HSPA8	3.45	759.01	7.78	220.28	0.03331
ORC1	314.42	1.56	-7.66	202.09	0.034891
CTD-2384A14.1	288.04	1.43	-7.65	201.43	0.042778
CPA6	339.19	1.78	-7.57	190.47	0.041548
SNHG14,SNORD116-20	3.94	742.34	7.56	188.44	0.00707
FAT3	358.09	2.16	-7.37	165.57	0.015002
KIAA0556	2594.11	22.18	-6.87	116.98	0.00707
ATP6V1G2,ATP6V1G2-DDX39B,DDX39B	3.63	370.03	6.67	101.83	0.00707
BRINP3	225.98	2.63	-6.43	85.95	0.0407
RPL21	8.09	665.14	6.36	82.26	0.012779
RP11-677M14.7	173.09	2.15	-6.33	80.51	0.015002

The top panel heatmap seen in Figure 6-5 shows the visual representation of Table 6-5, which describes the top 20 RNAs with a low and high expression between acute TIH samples and C1 control samples.

The bottom panel heatmap of Figure 6-5 shows the relative impact of changes in gene expression within each population (acute TIH. samples and C1 control samples). Within acute TIH samples, *KIAA0556* and *C17orf64* were highly expressed RNA's, however when compared to C1 control expression, *KIAA0556* and *C17orf64* were lower in C1 controls.

EEF1A1 (part of a complex to deliver tRNAs to the ribosomes), *RNF39* (encodes a protein part of Major Histocompatibility Complex, class I) and *MED27* (a transcription activator) were lowly expressed in the acute TIH samples, when comparing the relative expression in the C1 control samples.

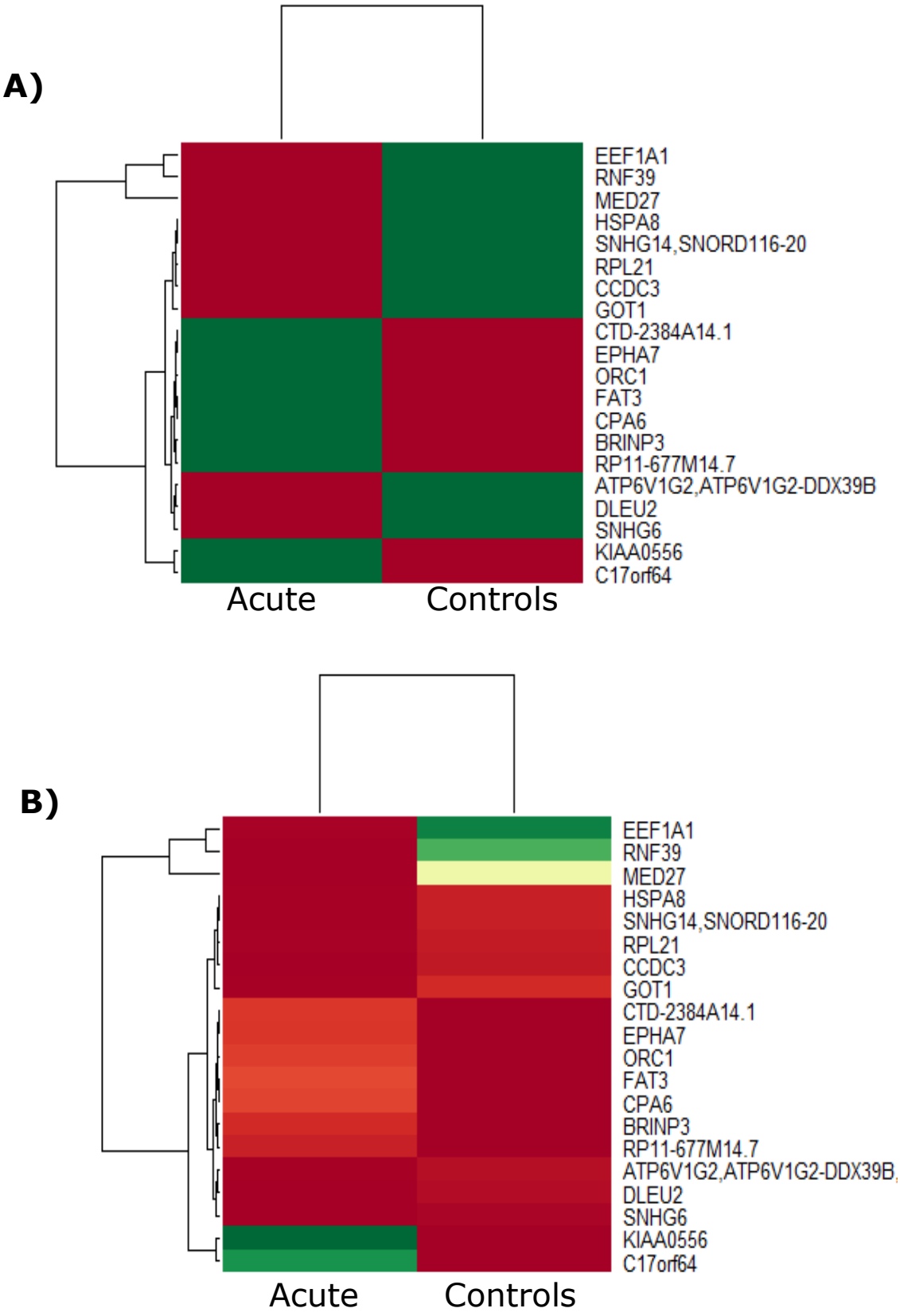


Figure 6-5 Heatmap of the top 20 differential expression genes between acute TIH and control samples. *Red =lowly expressed mRNA, Cream = mid-expression mRNA Green = highest expressed mRNA. A) The heatmap shows the standard differential expression, as seen in the table. B) This heatmap shows differential expression once the columns have been normalised within the column/population.*

6.2.5.1.3 Convalescent TIH vs C2 controls

Table 6-6 shows the comparison between Convalescent TIH samples and C2 controls 11 non-coding RNAs e.g. *SNHG6, RP11-337L12.1* or *RP11-527D15.1* and 9 coding mRNAs e.g. *ERH* (Enhancer Of Rudimentary Homolog), *RLF* (Rearranged L-myc Fusion) and *FGD1* (Faciogenital Dysplasia 1). These specific protein-coding genes are involved in transcriptional regulation. *CCDC3* was expressed in Acute TIH samples and C1 control samples. As with the other comparisons, no renal electrolyte transporters are found within the top 20 genes.

Table 6-6 Top 20 genes expressed between Convalescent TIH and C2 controls when ordered by fold change. *The top 20 genes are listed by fold change. The log2 (fold change) is also listed to show direction.*

Gene name	Convalescent FPKM	C2 Control FPKM	log2 (fold_change)	Fold change (2^_)	q-value
RP11-337L12.1	0.03	1216.27	15.15	36477.01	0.024865
SNHG6	1448.16	0.046444	-14.93	31181.44	0.000477
RLF	0.21	2064.78	13.265	9843.80	0.006662
DDX26B-AS1	0.15	695.265	12.16	4590.71	0.022989
RP1-212P9.2	1902.77	0.42225	-12.14	4506.21	0.018244
CLN5	0.18	539.88	11.56	3017.84	0.005572
SMIM13	0.52	1354.09	11.36	2628.09	0.021205
LRRC56	0.21	382.148	10.85	1840.40	0.019001
RP11-429P3.5	1.90	3046.77	10.65	1606.16	0.00253
RNU6-707P, RP11-38O23.4	299.85	0.205251	-10.51	1460.96	0.033458
RP11-10N16.3	1.06	1428.43	10.39	1344.92	0.01346
U1	5095.43	4.19758	-10.25	1213.87	0.004453
C9orf57	1955.05	1.69382	-10.17	1154.22	0.004039
KRT40	0.70	732.552	10.02	1041.68	0.002752
ERH	5.73	5769.12	9.97	1006.03	0.000477
RAET1G	0.85	805.621	9.89	949.41	0.003418
RP11-527D15.1	0.41	352.63	9.76	867.85	0.017739
CCDC3	0.52	441.414	9.72	842.16	0.000477
RNA5SP387	88082.30	112.849	-9.61	780.53	0.016471
FGD1	776.07	1.02067	-9.57	760.36	0.009761

Figure 6-6 shows the Heatmaps between convalescent TIH samples and C2 controls, with the top panel heatmap visually representing the results from Table 6-6 and the bottom panel heatmap the changes in gene expression within each population (convalescent TIH samples and C2 controls samples). Only *RNA5SP387* was the highest expressed RNA in convalescent TIH samples, but highly expressed in the C2 control samples, however a significantly increased expression of *ERH* was seen in C2 controls, but not in convalescent samples.

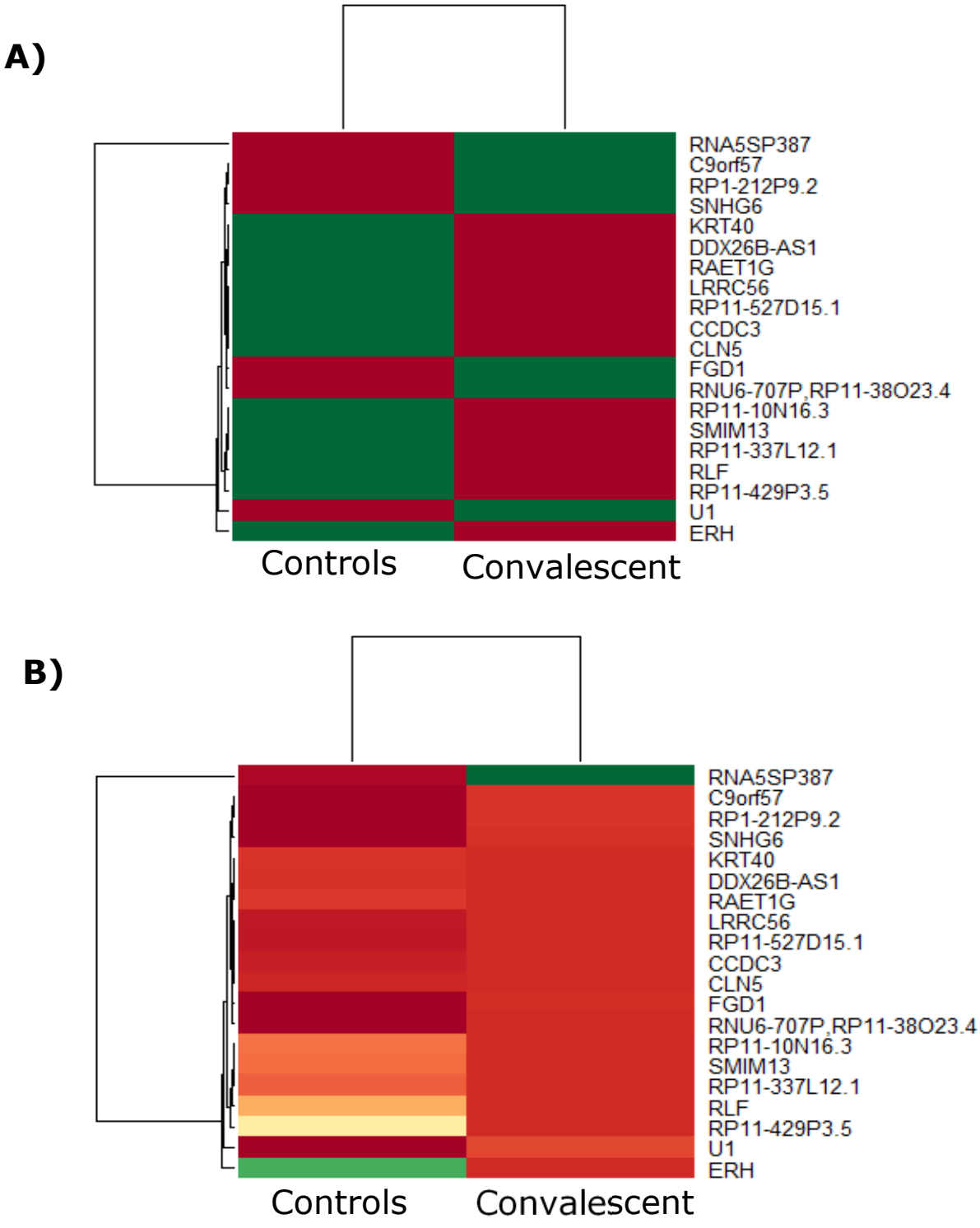


Figure 6-6 Heatmap of the top 20 differential expression genes between convalescent TIH and control samples. *Red =lowest expressed mRNA, Cream = mid-expression mRNA Green = highest expressed mRNA. A) The heatmap shows the standard differential expression, as seen in the table. B) This heatmap shows differential expression once the columns have been normalised within the column/population.*

6.2.5.2 Differential expression by directional fold change

As with fold change, the same comparison groups were identified by their directional change, according to the greatest significant change. In each comparison, the top 10 upregulated (values with a positive value) and the 10 downregulated (values with a negative value) were listed. Significance was determined by the q-value (corrected p-value) to reduce the chances of false positives. It was vital to include analysis with directional fold change as this prevents the exclusion of those genes of both directional effects.

With the comparison between Acute TIH and Convalescent TIH samples, no additional genes were identified, therefore no table has been included (Refer to Table 6-4). When performed directional fold change analysis between Acute TIH samples and C1 control samples, only 1 gene was identified in addition to fold change analysis; this is listed below.

Table 6-7 Additional RNAs identified by directional fold change analysis between Acute TIH samples vs C1 control samples. *ACBD7 is the only gene from the top 20 directional fold change analysis, which was not identified by fold change analysis, but would have been excluded if directional fold change were not performed.*

Gene name	Acute FPKM	C1 Control FPKM	log2 (fold_change)	Fold change (2^_)	q-value
ACBD7	557.64	6.94	-6.33	80.37	0.00707

Table 6-8 shows an additional 3 genes identified from directional fold change analysis between convalescent TIH and C2 controls.

Table 6-8 Additional RNAs identified by directional fold change analysis between Convalescent TIH samples vs C2 control samples.

Gene name	Convalescent FPKM	C2 Control FPKM	log2 (fold_change)	Fold change (2^_)	q-value
SPTY2D1	591.57	1.05	-9.14	562.41	0.000477
RNA5SP74	21827.2	38.65	-9.14	780.53	0.000477
HSPA8	827.11	1.33	-9.27	620.46	0.001394

All RNAs identified from fold change and directional fold change have been combined and used to create a Venn diagram, comparing those RNAs in common between comparison groups.

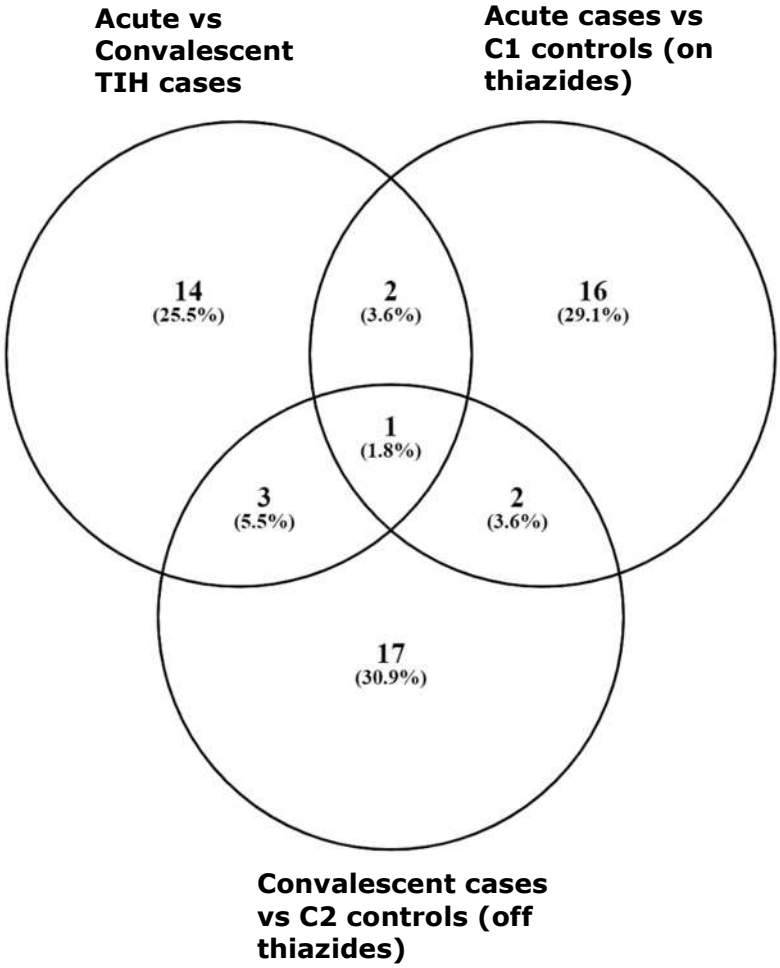


Figure 6-7 Combined differential expressed RNAs after fold change and directional fold change analysis. *The Venn diagram shows the genes that are common and different between Acute TIH vs Convalescent TIH, Acute TIH vs C1 controls and Convalescent TIH vs C2 controls.*

The Venn diagram in Figure 6-7 shows the common genes after the combination of RNAs from fold change and directional fold change. The total combined number of RNAs for Acute TIH vs Convalescent TIH samples was 20. The Acute TIH vs C1 controls had a combined RNAs number of 21 and the combined number of RNAs for convalescent TIH and C2 controls was 23.

The common genes between all comparison groups was SNHG6. The common genes between acute vs convalescent and acute vs C1 controls were CPA6 and EPHA7. The common genes between acute vs convalescent and convalescent vs C2 controls were FGD1, C9orf57, and RP11-429P3.5. The common genes between acute vs C1 controls and convalescent vs C2 controls CCDC3 and HSPA8.

6.2.5.3 Identifying genes based on population expression patterns

Using Cuffdiff results from sections 6.2.5.1, 6.2.5.2, and results from Cuffnorm command, heatmaps were generated to identify true population expression from the top 20 RNAs from fold change and directional fold change. By normalising across the row (by RNA), the heatmaps are also able to identify specific samples are driving the differential expression observed in the results above. RNAs with more than half of the samples displaying upregulation (blue) were considered a true representation of differential expression within the population.

6.2.5.3.1 Heatmap of Acute TIH and Convalescent TIH samples

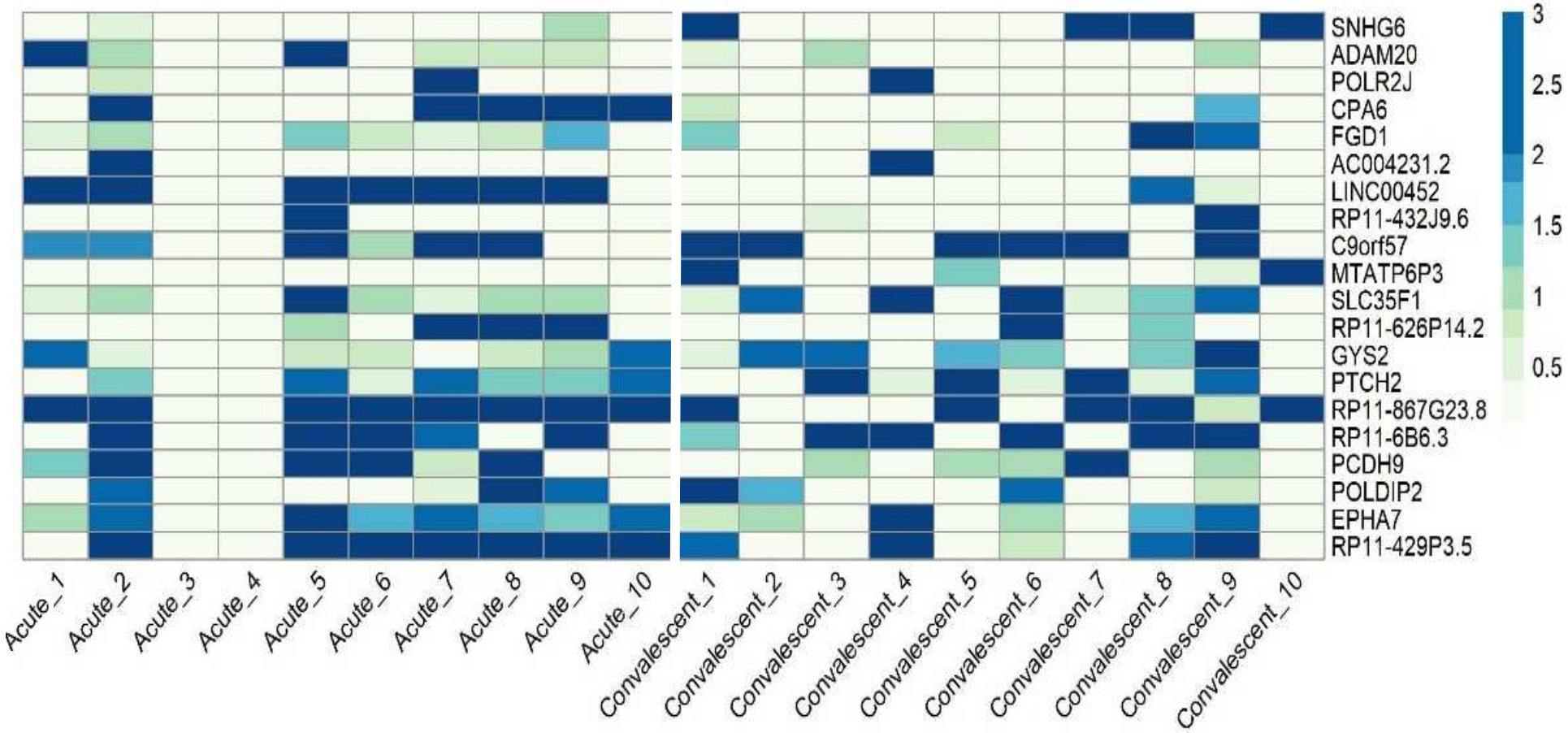
Figure 6-8 shows the top differentially expressed genes individual acute TIH and convalescent TIH patient samples. This heatmap describes the top differentially expressed RNAs in each patient sequenced, allowing identification of those, which are driven by one or two samples. It also provides a visual representation of the sequencing data to identify any biological changes between acute TIH and convalescent TIH conditions.

Samples Acute 3 and Acute 4 did not produce any FPKM values in the top differentially expressed genes, but upon closer inspection of each individual sample, genes were identified in these samples. It is extremely likely that Acute 3 and Acute 4 were subjected to a high level of degradation in comparison to the other samples.

LINC00452, GYS2, EPHA7 are RNAs that are true representatives of differentially expressed genes between acute TIH and convalescent TIH samples. *LINC00452* shows a clear upregulation in acute TIH samples across most samples (Acute samples 3, 4, and 10 do not show any expression) when compared to the convalescent samples. *EPHA7* is also upregulated across acute TIH samples, in contrast to convalescent TIH samples. Whilst not as strongly expressed as *LINC00452*, *GYS2* does show upregulation across most of convalescent TIH samples, when compared to acute TIH samples.

SNHG6, ADAM20, POLR2J, and *AC004321.2* were amongst the RNA's excluded from the top differentially expressed. *SNHG6* was excluded due to the lack of a majority of the convalescent samples not expressing the RNA. *ADAM20* is an example of one of the RNAs excluded when only 2 acute samples out of 10 were highly expressing the RNA. *POLR2J* and *AC004321.2* were excluded due to having no biological response between the conditions.

Figure 6-8 Heatmap of top differentially expressed RNAs in individual samples of Acute TIH and Convalescent TIH patients. *The heatmap shows a visual representation of the Cuffnorm data in each patient sample in order to identify true expression across the population. Blue represents the high expression, whilst cream represents no expression.*



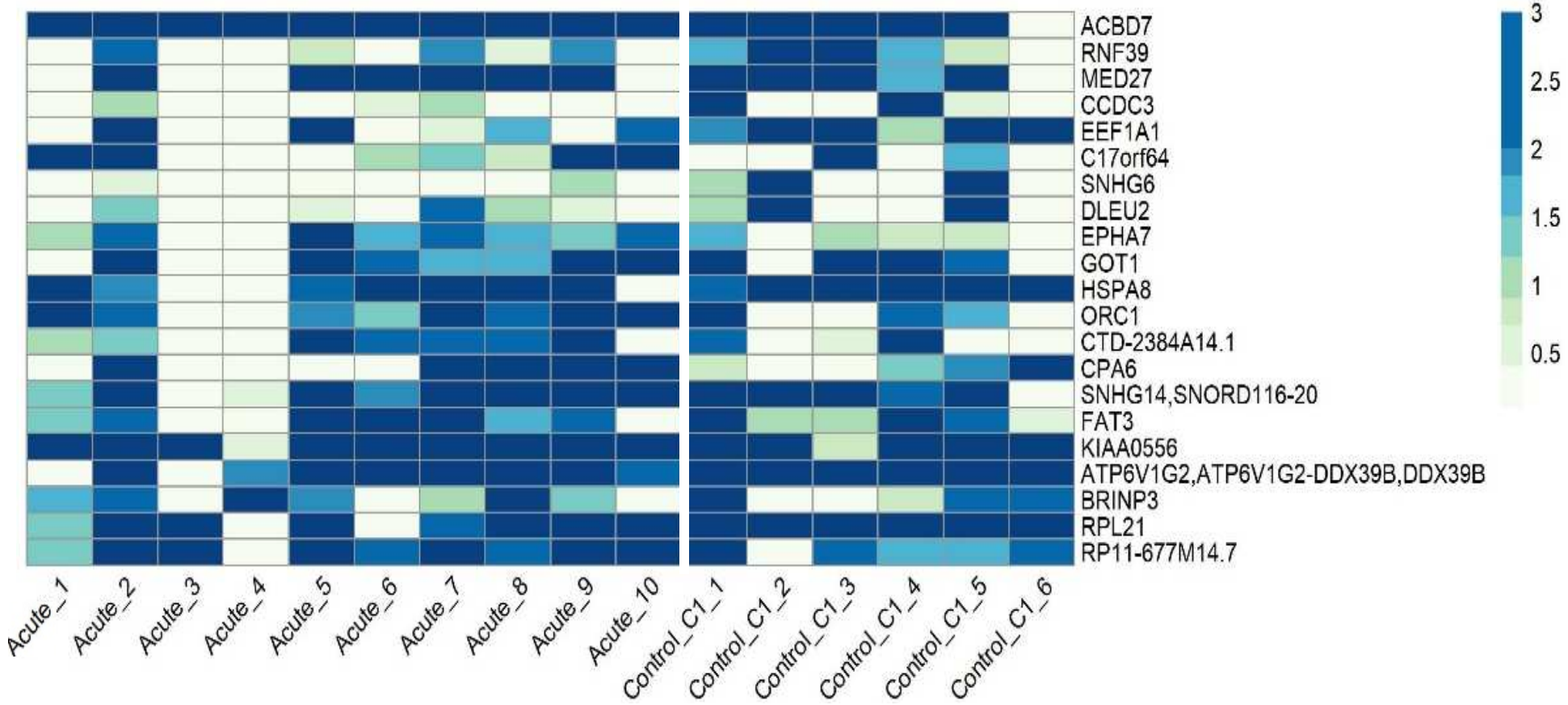
6.2.5.3.2 Heatmap of Acute TIH and C1 control samples

Due to poor quality and low percentages alignment, four of the C1 control samples were removed from RNA-sequencing analysis, as described in Section 6.2.2. Unlike with the Acute and Convalescent comparison, there was, therefore, an unequal number of samples for each condition.

Figure 6-9 shows that very few RNAs are differentially expressed across the population. Once normalised by row RNAs like RNF39, *EEF1A1* and *CTD-2384A14.1* were identified, as truly differentially expressed. *RNF37* and *EEF1A1* were upregulated in C1 controls but downregulated in acute TIH samples, whilst *CTD-2384A14.1* was upregulated in the acute TIH samples but downregulated in the C1 control samples. Their functionality is discussed below.

Amongst the RNAs that were removed from the list of the top differentially expressed were *ACBD7, MED27, FAT3*, and *HSPA8*. After normalisation across the row, *ACBD7, MED27, FAT3,* and *HSPA8* showed no change in expression between the two conditions when the data from the Cuffnorm command was applied.

Figure 6-9 Heatmap of top differentially expressed RNAs in individual samples of Acute TIH patients and C1 Controls. *The heatmap shows a visual representation of the Cuffnorm data in each patient sample in order to identify true expression across the population. Blue represents the high expression, whilst cream represents no expression.*



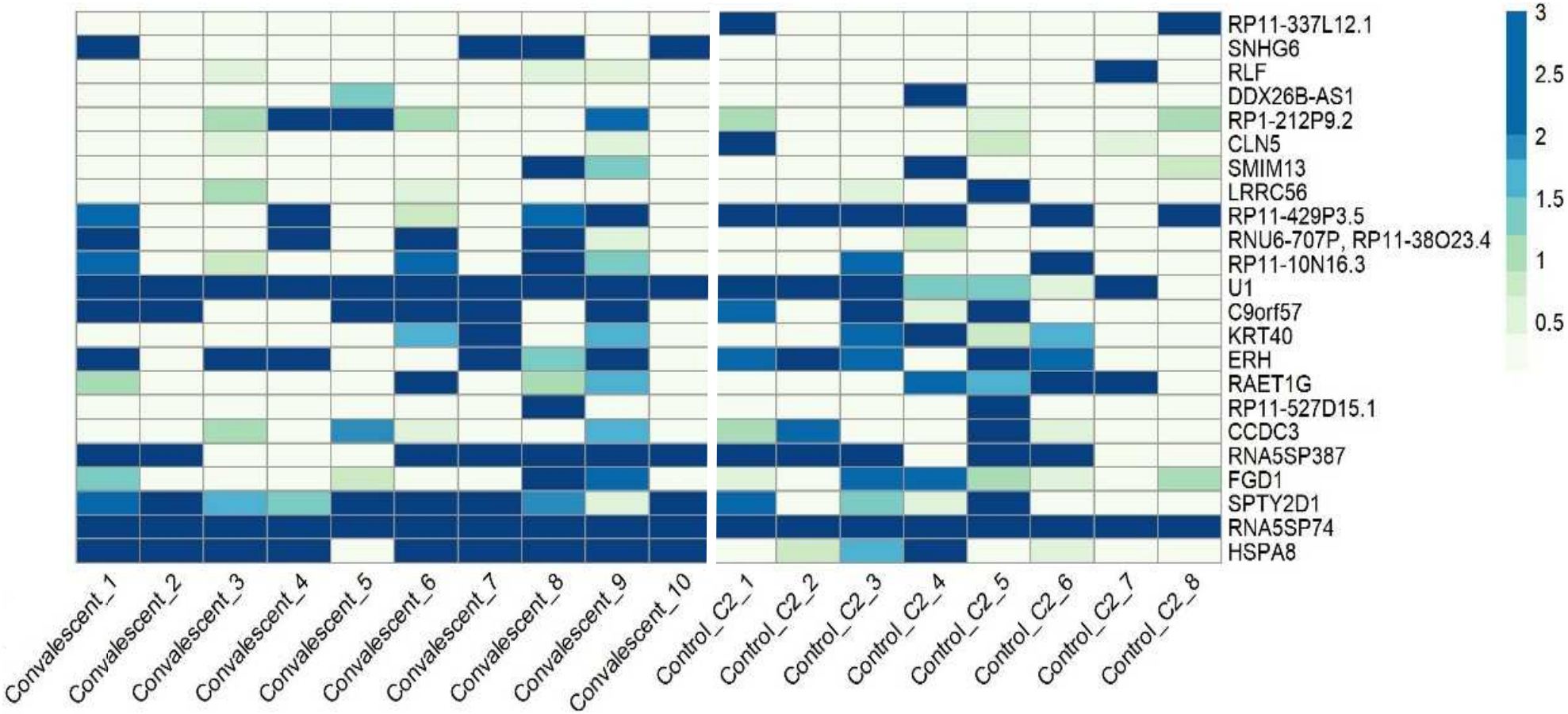
6.2.5.3.3 Heatmap of Convalescent TIH samples and C2 control samples

As with the comparisons between acute TIH samples and C1 controls, poor quality and low percentages alignment resulted in the removal of two of the C2 control samples from RNA-sequencing analysis, as described in Section 6.2.2.

Figure 6-10 showed many of the top differentially expressed genes were not truly differentially expressed, as suggested when first identified after initial Cuffdiff analysis. This is because only one or two samples were driving the high Cuffdiff values and therefore the decision was made to remove them from the final list of differentially expressed genes between Convalescent TIH and C2 controls. When producing the heatmap, normalisation across the row showed little difference between conditions, as seen with Acute TIH and C1 control comparisons. There were also some RNAs that were only expressed in one or two samples, therefore not showing a true representation of the population. Examples of those RNAs included *RP11-337L12.1*, *RLF,* and *CLN5*.

HSPA8 and *SPTY2D1* were the few RNAs remaining as differentially expressed across the population. Both *HSPA8* and *SPTY2D1* showed a high upregulation of the RNA in convalescent TIH samples, but downregulation in the majority of the C2 controls. There were no RNAs upregulated on in the C2 controls but downregulated in the convalescent TIH samples. Functions of the remaining differentially expressed RNAs are discussed in Section 6.2.5.4.

Figure 6-10 Heatmap of top differentially expressed RNAs in individual samples of Convalescent TIH patients and C2 controls.
The heatmap shows a visual representation of the Cuffnorm data in each patient sample in order to identify true expression across the population. Blue represents the high expression, whilst cream represents no expression.



6.2.5.4 Differentially expressed genes representative of the sample population

Data from Cuffdiff and Cuffnorm analysis have been used to produce the tables below displaying the final RNA's which are significantly differentially expressed, representative to the population. Table 6-9 shows the RNA's from the comparison between acute TIH and convalescent TIH, with their associated functions. No RNA's listed in Table 6-9 have been reported to have associations with TIH, but they are involved in the regulation of cellular biology e.g. *CPA6* is involved in the production of amino acids, whilst *PTCH2* may be involved in the regulation of cell division due to its connection with the Hedgehog Pathway [195]. *GYS2* is the only listed RNA that may be associated with TIH. Four RNAs listed are non-coding or uncharacterised.

Table 6-9 Differentially expressed RNA's between acute TIH and convalescent TIH samples. *The RNA's listed below are a true representative of differentially expressed genes across the population, with many of the original top genes removed due to the drive of one or two samples. Functions of each RNA's have been included, along with repeating the data from Cuffdiff analysis.*

Gene name	Acute FPKM	Convalescent FPKM	log2 (fold_change)	q-value	Function
CPA6	373.42	0.26	-10.47	0.010929	• Digestion of food • Synthesis of neuroendocrine peptides
LINC00452	358.93	0.30	-10.22	0.021916	• Long Intergenic Non-Protein Coding RNA
C9orf57	1.81	1869.59	10.01	0.002078	• Uncharacterised Protein
SLC35F1	0.95	674.83	9.48	0.001582	• Uncharacterised Protein (also known as C6orf169)
GYS2	0.88	458.65	9.03	0.000428	• Catalyses the synthesis of glycogen from glucose
PTCH2	1.20	552.61	8.85	0.000428	• A tumour suppressor in the hedgehog signalling pathway
PCDH9	253.31	0.92	-8.10	0.000428	• Involved in cell adhesion in neural tissue
EPHA7	306.43	1.13	-8.08	0.001072	• Modulating cell-cell adhesion and repulsion
RP11-429P3.5	455.04	1.91	-7.90	0.001072	• Uncharacterised Protein

After the analysis from the heatmap, the majority of the RNAs were removed as the top differentially expressed RNAs, due to certain samples driving the differential expression or due to no effect observed. The majority of the RNAs listed in Table 6-10 were non-coding RNAs or not yet classified. The RNAs that are protein-coding (*RNF39, EEF1A1* and *EPHA7)* have no known associations with the TIH condition.

Table 6-10 Differentially expressed RNA's between acute TIH and C1 control samples. *The RNA's listed below are a true representative of differentially expressed genes across the population, with many of the original top genes removed due to the drive of one or two samples. Functions of each RNA's have been included, along with repeating the data from Cuffdiff analysis.*

Gene name	Acute FPKM	C1 Control FPKM	log2 (fold_change)	q-value	Function
RNF39	0.78	9394.38	13.55	0.0407	• Part of the MHC class I region
EEF1A1	8.76	10648.00	10.25	0.00707	• Involved in the delivery of tRNAs to ribosomes
C17orf64	2357.87	2.98	-9.63	0.015002	• Uncharacterised Protein
DLEU2	0.61	311.45	9.01	0.028204	• Long alternative non-coding RNA
EPHA7	283.17	0.84	-8.39	0.042604	• Modulating cell-cell adhesion and repulsion
CTD-2384A14.1	288.04	1.43	-7.65	0.042778	• Long Intergenic Non-Protein Coding RNA

The original list of differential expressed RNAs between convalescent TIH and C2 controls has been significantly reduced after combining data from Cuffdiff and Cuffnorm to create the heatmap seen in Figure 6-10. Table 6-11 show the only four RNAs, which remain differentially expressed and truly represent the population between the two conditions. Two of the four genes (*RNU6-707P, RP11-38O23.4* and *RP11-10N16.3*) are uncharacterised, meaning that although its locus has been identified, the protein it codes for has yet to be defined. The other two differentially expressed genes (*SPTY2D1* and *HSPA8*) are protein-coding but have no known associations with TIH.

Table 6-11 Differentially expressed RNA's between convalescent TIH and C2 control samples. *The RNA's listed below are a true representative of differentially expressed genes across the population, with many of the original top genes removed due to the drive of one or two samples. Functions of each RNA's have been included, along with repeating the data from Cuffdiff analysis.*

Gene name	Convalescent FPKM	C2 Control FPKM	log2 (fold_change)	q-value	Function
RNU6-707P, RP11-38O23.4	299.85	0.21	-10.51	0.033458	• Uncharacterised Protein
RP11-10N16.3	1.06	1428.43	10.39	0.01346	• Uncharacterised Protein
SPTY2D1	591.57	1.05	-9.14	0.000477	• Required for chromatin refolding involving histones
HSPA8	827.11	1.33	-9.27	0.001394	• Facilitates correct protein folding

6.2.6 Candidate mRNA approach

Using the current understanding of the pathophysiology of TIH, a candidate approach was also used to investigate those mRNA's thought to be involved in TIH pathology.

Table 6-12 describes SLC12A3 (NCC), SLAC9A3R1 (Na^+/H^+ exchange regulatory cofactor) and *SLC5A2* (sodium/glucose cotransporter 2), which are differentially expressed between acute TIH samples and convalescent TIH samples from CuffDiff results. SLC12A3 is expressed significantly more in acute samples compared to convalescent samples (4.53-fold change). *SLC12A3* was not differentially expressed between the acute vs C1 controls or convalescent vs C2 controls comparison groups.

SLC9A3R1 is a cofactor for the Na^+/H^+ exchanger 3 and shown to be significantly more expressed in the acute TIH samples by 17.03-fold.

Table 6-12 Candidate genes differentially expressed between Acute TIH samples and Convalescent TIH samples.

Gene Name	Acute FPKM	Convalescent FPKM	log2(fold_change)	Fold Change
SLC12A3	6.87	1.51	-2.18	4.53
SLC9A3R1	196.05	11.51	-4.09	17.03
SLC5A2	0.27	3.13	3.52	11.48

Whilst no candidate genes were significantly differentially expressed between acute TIH samples and C1 controls, *SLC9A3R2* was differentially expressed between convalescent samples and C2 controls by a fold change of 6.25 (Table 6-13). *SLC9A3R2* is the other cofactor of the Na^+/H^+ exchanger 3. No other candidate genes were differentially expressed between convalescent TIH samples and C2 control samples.

Table 6-13 Candidate genes differentially expressed between Convalescent TIH samples and C2 control samples.

Gene Name	Acute FPKM	Convalescent FPKM	log2 (fold_change)	Fold Change	q-value
SLC9A3R2	8.68	1.39	-2.64	6.25	0.019864

Whilst Cuffdiff produced results showing *SLC12A3* and *SLC9AR1* as differentially expressed, upon a closer analysis of the Cuffnorm data generated, it was discovered that only one sample was driving the high FPKM value seen in Table 6-9. Therefore, *SLC12A3 and SLC9A3R1* are probably not differentially expressed between Acute TIH and Convalescent TIH samples. However, *SLC5A2* remains a differentially expressed gene representative of the population.

Other genes that have previously been described in the literature as being associated with TIH e.g. *KCNJ1* (ROMK), *SLCO2A1* (PGT) and *SCNN1A* (ENaC), were not significantly differentially expressed between comparison groups.

Whilst they have never been implicated in TIH pathology, *WNK1*, *WNK4*, *CUL3* and *KLHL3* do regulate NCC, as seen in previous chapters. These genes coding for these proteins are also not differentially expressed. Although *WNK1 and* WNK4 were expressed at low levels, *CUL3* and KLHL3 were not expressed in any samples. This may suggest that as NCC is differentially expressed between acute and convalescent TIH samples, the protein's expression changes through another regulatory pathway or that WNK1/4, CUL3 and KLHL3 mRNA is not well expressed in urinary exosomes.

6.3 Discussion

This analysis was split into two methods: hypothesis-free and the candidate approach. The hypothesis-free method looked at the differential expression of all genes by fold change and in order of statistical significance of the difference in mRNA expression, regardless of their known or predicted role in molecular mechanisms underlying TIH. The candidate approach showed results by selecting genes/proteins, which have been linked to the pathology of TIH or molecular pathways thought to be involved in the condition.

Overall, it is likely there has been a significant amount of RNA degradation due to some samples not expressing any of the top differentially expressed genes, which limits the value of these data. The results have also shown a number of samples driving the differential expression between conditions, therefore not showing a true representation of the population expression. The chapter has identified those RNAs that do show true differential expression.

6.3.1 The use of the Hypothesis Free Approach

The hypothesis-free approach aims to identify the most differential expressed genes between two certain conditions. In this case, the top 20 fold change genes and the top 20 directional fold change genes were used to identify any known genes related to TIH, although in this analysis the results from each list were very similar if not identical. It was also used to potentially identify any genes yet to be associated with the condition. Using the fold change method shows the difference in expression between each patient group, regardless of direction, whilst directional fold change includes those RNAs that are upregulated and downregulated.

Unfortunately, many exosomal RNAs were removed from the top differentially expressed RNAs due to specific samples driving the differential expression. It is unclear why some samples expressed significantly more of a specific RNA than other samples. One possible reason for differences in exosomal RNA expression between samples could be degradation [196]. One report has already suggested exosomes contain enriched degraded mRNA products, suggesting this high concentration of degradation is part of gene regulation

[196]. The uncharacterised genes and pseudogenes reported above maybe products of mRNA degradation within epithelial cells.

Due to reports suggesting exosomes are a rapid process to correct gene expression and from the results of uncharacterised genes in this chapter, RNA expression within exosomes must be compared to the RNA expression within the kidney. Only through this comparison will we be able to confirm if the results presented are representative of physiological function or if RNA expression has been subjected to degradation [197].

Overall, the hypothesis-free approach did not identify any mRNAs, which were significantly differentially expressed in acute TIH or convalescent TIH patients compared to controls.

6.3.2 Candidate gene approach

Although the candidate gene approach originally produced RNA's related to TIH and this thesis, upon closer analysis using Cuffnorm data, it was concluded that RNA's *SLC12A3, SLC9A3R1,* and *SLC9A3R2* were driven by only one patient sample and therefore not representing the overall population. These RNAs were removed from the final results.

Although not reported to be associated with Thiazide-Induced Hyponatraemia, *SLC5A2* (a sodium/glucose transporter) was identified as differentially expressed through the population. The RNA is of interest, due to the relationship between glucose and sodium within the condition.

As with the Hypothesis free approach, the Candidate gene approach did not produce any RNAs associated with Thiazide-Induced Hyponatraemia.

6.3.3 Glucose-related proteins

During the hypothesis-free and candidate approach analysis, glucose related proteins were identified as significantly differentially expressed between acute and convalescent TIH samples and with control samples.

A recent cohort study by Ware *et* al. has shown acute TIH patients have an increased glucose concentration (7.6±0.4mM) compared to convalescent (5.9±0.2mM) and their associated controls (6.1-6.5±0.2-0.4mM) [77]. Comparing these results with the results from the RNA-sequencing analysis of this chapter may provide an explanation for the higher glucose concentrations in acute TIH patients.

GYS2 and *SLC5A2* were differentially expressed genes between acute and convalescent TIH samples, with acute TIH samples expressing less *GYS2* and *SLC5A2* than convalescent TIH samples.

GYS2 is activated with increased glucose concentrations to create links between UDP-glucose, forming glycogen [198]. Considering *GYS2* function and the low expression in acute TIH samples, it may be suggested that increased glucose concentrations are observed because of the decreased expression of *GYS2*, therefore glucose remains within the blood. However, according to Protein Atlas, its protein, GYS2, is reported only to express in the liver, unlike *GYS1,* which is expressed in several organs including the kidney [198]. In this case, it may be speculated that *GYS2* mRNA does not reach translation and therefore not endogenously expressed. Further qRT-PCR experiments would be required to compare *GYS2* expression in kidney and liver tissue to confirm this RNA sequencing result.

6.3.4 Summary

Data produced in this chapter is the first of its kind to identify RNAs associated with Thiazide Induced Hyponatraemia by RNA-sequencing individual urinary exosomal samples. Despite using the Hypothesis-free and the candidate free method, no reported RNAs were identified. Although *SLC12A3* and *SLC9A3* cofactors were reported as differentially expressed from the Cuffdiff command, further analysis with the Cuffnorm command provided evidence of one sample driving the results from Cuffdiff.

The relationship between glucose and sodium in TIH has been well reported in peer review papers, but the cause of the relationship remains unclear. This chapter identified *GYS2* and *SLC5A2*, which may suggest a possible link to the glucose and sodium relationship within the condition. However, further pathway analysis is required to show any associations.

Although RNase inhibitor and the Seramir RNA extraction method were used, samples showed signs of degradation. Due to the degree of degradation, it cannot be determined if exosomes can be used to reliably sequence mRNA/genes. From the results of this chapter, we have been unable to identify RNAs associated with Thiazide Induced Hyponatraemia.

Chapter 7: Generalised Discussion and Conclusion

7 Generalised Discussion and Conclusions

7.1 Hypothesis and Aims

Essential hypertension is increasing across the worldwide population with approximately 30% diagnosed with the condition. Discovering the causes of essential hypertension is vital in order for the correct treatment to be given. NCC and its related proteins have been associated with regulating blood pressure due to genetic diseases like Gordon and Gitelman syndrome. This thesis focused on the characterisation of NCC and its related proteins. Through the extraction of urinary and media exosomes, NCC's characteristics were further explored in association with blood pressure and associated diseases. The experiments presented in this thesis aimed to show the importance of urinary exosomes, as well as how they can be used to measure gene and protein expression of the sodium-chloride cotransporter. This thesis addressed the following areas:

- Optimising extractions of:
    - Exosomes from the urine of patients
    - Exosomes from cell culture media
    - Protein from exosomes
    - mRNA from exosomes
- Conducting a physiological study of the relationship between serum K^+ and 24hr dietary K^+ intake and the exosomal expression of NCC, pNCC, WNK4 and SPAK in the kidney
- Identifying target mRNA in urinary exosomes using the optimum extraction method
- Presenting differential expression of *SLC12A3* and related genes between healthy and TIH participants
- Studying communication of NCC-rich exosomes between HEK293 and primary epithelial Proximal Tubule Kidney cells.

7.2 Overall findings

7.2.1 Urinary exosomes can be used for measuring mRNA and protein expression

The kidney is an organ, which can fluctuate biochemical concentrations of electrolytes within the body through reabsorption of electrolytes, consequentially affecting key systems within the body like blood pressure or water retention. However, the ability to study the current cellular processes in the kidney in order to identify how electrolyte reabsorption is affected is extremely difficult. Fortunately, the discovery of exosomes within urine has created an opportunity to increase the understanding of the molecular functions of the kidney without the need for renal biopsies [199].

This thesis has shown the versatile uses of human urinary exosomes. These extracellular vesicles have provided molecular and proteomic information from the kidney's nephrons. Protein expression of all 3 isoforms of NCC and phosphorylation sites most associated with its activity were successfully measured using urinary exosomes and correlated with electrolytes reported to change blood pressure.

Isolated urinary exosomes were also used to extract exosomal mRNA using the Seramir extraction method. Providing the quality of the mRNA is suitable, urinary exosomal mRNA can be sequenced, identifying key genes in various conditions.

7.2.2 Potassium's effect on pNCC expression and regulation of NCC

Chapter 4 showed associations between NCC and its related proteins and electrolytes based on previous literature. Although serum potassium did not correlate with NCC_3 and $NCC_{1/2}$, the electrolyte did negatively correlate with phosphorylated NCC S91 and T60. This suggests that serum potassium has an association with type 2 NCC regulation (activity), but no association with type 1 NCC regulation (expression). It can be concluded that as serum potassium concentrations increase, phosphorylation of NCC reduces and so does its activity.

Amongst other electrolytes that were correlated with NCC_3, serum chloride was negatively correlated with NCC_3 protein expression, concluding that the electrolyte regulates Type 1 NCC regulation.

The results from chapter 4 did not find correlations between 24hr urinary potassium excretion and phosphorylated NCC, however, differences between published reports and data presented in this thesis could be due to the different methods of measuring potassium intake vs excretion. Controlling and measuring potassium intake prior to collecting urine samples would perhaps show the true dietary and protein expression of each patient's individual potassium intake, which was the aim of the chapter.

7.3 Limitations

The main limitation of this study was the inability to fully identify and quantify exosomes. Although biomarkers were used that were specific to the nephron of the kidney, it was impossible to fully guarantee that the samples used in these experiments were pure exosome fractions due to the possibility of microvesicles and apoptotic bodies [92].

Lack of facilities also prevented the ultracentrifugation extraction method of exosomes. Ultracentrifugation, specifically differential ultracentrifugation, has shown to extract higher quantity and quality exosomes compared to ultrafiltration and exosome bind chemicals. If the facilities were available, Chapter 5 would have benefitted from also including ultracentrifugation extractions to confirm if this type of extraction is the best.

The ability to be able to identify and quantify exosomes would have helped with normalising mRNA and protein expression. However, this requires specific equipment and knowledge, which was not available at the time of mRNA and protein experiments.

Whilst using urinary exosomes can give an indication to the transcriptomics and proteomic functions of an individual cell, it is not possible to confirm this without also comparing to kidney tubule tissue. Unfortunately, accessing human kidney tissue through biopsies is extremely difficult in this thesis due to the highly invasive procedure and the complications that may follow. Therefore, gaining human kidney tissue for normonatraemic hypertensive patients or acute/convalescent TIH patients would have been impossible. However, studies that have use of kidney tissue would be advised collecting spot urine samples to allow comparisons between urinary exosomes and cellular biology. Fundamentally, it is the theory needs to be confirmed, so it would be possible that an animal study could be designed using kidney tissue and urinary exosomes, where mRNA and protein expression can be compared. In order to fully compare between exosomes and its tissue of origin, transcriptomics (RNA sequencing) and proteomic studies should be performed, as this will give a broader range cellular outputs than just looking into the specific genes of interest.

These studies could also identify the molecular role for urinary exosomes, as excess exosomal mRNA and protein expression may indicate exosomes are used as a disposal system.

It must also be mentioned that although RNA sequencing was able to generate a database of expressed RNAs and quality control checks were performed to prevent any errors, the urinary exosomal RNA was of poor quality despite attempts to improve the methodology between Chapters 5 and 6. The best extraction method was identify and RNase inhibitor was included at all possible stages of extraction, however the RNA data in Chapter 6 did show a high level of degradation. RNase inhibitor could be added at the same time as the addition of the protease inhibitor, however the downside to this, is RNase inhibitor is significantly more expensive, and therefore may be economically impractical. The method of extracting exosomes and exosomal RNA could be improved. Unfortunately, the resources to compare between ultrafiltration and ultracentrifugation were not possible, as reports suggest ultracentrifugation can improve quality and quantity [132]. More recently, new methods to quantify exosomes have the potential to improve RNA quantity and quality further. The use of the Nanosight LM10 instrument has been shown to extracted exosomal RNA of up to 15ng, which is significantly higher than RNA concentrations shown in this thesis.

The aim of Chapter 3 and 5 was to validate methodology, and therefore sample size would not have greatly affected the findings in these chapters. Despite using a power calculation to calculate the sample size for chapter 4, many of the findings were negative. It is difficult to precisely identify the reason for these negative results, as the aim of Chapter 4 was to measure protein in expression without controlling electrolyte intake, as well as the possibility that urinary electrolyte excretion is not a true representation of electrolyte intake. If this project were to be repeated, an increased sample size would be encouraged, which in addition to confirming if the correlations reported in this study were true, would also increase the effect size to above 0.5 e.g. a sample size of 40, would increase the effect size to 0.6 or more.

A power calculation may also have been necessary in Chapter 6, however, due to economic reasons, only 10 samples were selected for acute TIH, 10 samples for convalescent TIH and 20 samples for controls. If the sample size had been larger for this study, some genes may not have been removed due to one or two samples driving the differential expression. This may give a better representation of which genes are truly associated with TIH.

7.4 Proposed future work

7.4.1 Identifying urinary exosomal protein expression by quantitative methods

Whilst western blotting is the standard method for measuring exosomal protein expression, it has many disadvantages to the methodology. Firstly, western blotting may lack sensitivity, due to the need to rely on specific antibodies. The procedure is also time-consuming, due to the long antibody incubation times. When using western blotting for exosomal protein expression, it can often yield poor result when exosomes are low in abundance.

To improve the sensitivity and specificity of measuring exosomal protein expression, various methods are emerging to fully identify and quantify exosomal fractions. The identification and quantification of exosomes are becoming essential when working with these extracellular vesicles, because of the possibility of other vesicles and free molecules included within the samples.

An increasing number of experiments are avoiding western blotting and using nanoparticle tracking analysis for exosome to generate an exosome concentration, followed by flow cytometry to measure protein expression [200, 201].

Nano-tracking particle analysis is a newly developed semi-quantitative method for identifying exosomes, based on size measurement, using Nanosight instruments and NTA software for analysis [202]. The procedure quantifies and measures the extracellular vesicles after isolation (either ultracentrifugation, ultrafiltration or exosomal binding chemicals), providing

a concentration of exosome particles, which has not been possible to produce in this thesis [201]. With a concentration, it would be possible to normalise results from western blots. The nano-tracking particle analysis can also be used on any sample type (blood plasma, urine, media), allowing the identification and quantification of exosomes to become standardised, something which is currently lacking in exosomal research [203].

Multiplex flow cytometry assays are a new method to identify and quantify exosomal protein in one assay [203]. Polystyrene beads are bound to the exosomes to increase the overall size. The flow cytometer can now detect the bead-bound exosomes through emitting fluorescence. The addition of beads overcomes the challenge of binding of small exosomes. Multiple antibodies can also be bound to exosomes to measure protein expression e.g. CD63 or CD81, whilst also measuring surface expressed proteins e.g. NCC or AQP2 from the exosomes cellular origin [200, 201].

The flow cytometer as a quantifier and measurement of exosomal protein expression has been shown to be reproducible, due to the increase in use without the accompanying western blots. Although previous studies have only used exosomal markers, for example, CD63 and CD9, flow cytometry has yet to be used for measuring varied protein expression, like NCC and NKCC1/2.

The advantage of the multiplex flow cytometry assay is that there is a decreased chance of the antibodies binding to free proteins, which is a possibility in western blotting. This is because the assay relies on the binding beads to the exosomes generate an increased surface area, allowing for specific detection. The disadvantage is the exosomes must remain intact due to its dependence on sorting exosomes by specific markers. The technique also does not allow exosomes to be used in further experiments, and therefore this technique can only be used for analysis only.

7.4.2 Confirmation of exosomal NCC communication by confocal microscopy

Although the western blotting and light microscope data suggest exosomes and exosomal NCC transferred to the PKTC cells, this cannot be confirmed without the use of a confocal microscope. This type of microscopy is required to confirm the uptake and co-localisation of the HEK exosomes into the primary epithelial PKTC cell line. Although the PKTC were washed to remove any exosomes that were resting on the top of the PKTC cell membrane, an exosomal entry cannot be confirmed without confocal microscopy.

The confocal microscopy produces a greater resolution image due to the two lens that are used to focus on the same point, as well as only allowing light from the focus point to each the detector [204]. The confocal microscope and the accompanying software creates 3D images of the focal point, meaning cross-sections are taken of the sample [204].

Confocal microscopy has shown the uptake of proximal tubule exosomes in the distal nephron and collecting duct cells in a previous study [205]. The study shows kidney exosomes can be accepted into recipient cells and communicate with each other for example GAPDH from Proximal cells can regulate the functionality of ENaC (located in the collecting duct) [205, 206].

7.4.3 Exosomal pathway analysis between Acute and Convalescent TIH patients

Now that it has been discovered that mRNA can be successfully extracted from exosomes and analysed from RNA-sequencing, pathway analysis between acute and convalescent TIH patients could be performed. Pathway analysis can be used to further explore related genes/proteins and how they change in expression depending on the condition [207]. In relation to this thesis, pathway analysis could be used to confirm why *GYS2* expression decreases in acute TIH patients when compared to convalescent TIH patients.

7.5 Final Conclusions

The results presented in this thesis show the characteristics of NCC and its related proteins. Using exosomes, NCC gene and protein expression were measured using urinary exosomes. Phosphorylated NCC is associated with serum potassium, but not with dietary potassium or sodium, which could be linked with the activity of NCC's regulatory pathways. It has also been identified that urinary exosomes can be used to measure differential expression between acute and convalescent TIH patients, which could lead to future pathway analysis and a better understanding to those patients that are more predisposed to developing TIH. The work carried out in this thesis has commented on the advantages and disadvantages of working with exosomes, but it has also contributed to the growing knowledge of how these extracellular vesicles can be used to study the intracellular functions of cells without invasive biopsies.

8 References

1. McKee, J.A., et al., *Detection of Na^+ Transporter Proteins in Urine.* Journal of American Society of Nephrology, 2000. **11**: p. 2128-2132.

2. Esunge, P., *From blood pressure to hypertension: the history of research.* Journal of the Royal Society of Medicine, 1991. **84**: p. 621.

3. Glover, M., A.M. Zuber, and K.M. O'Shaughnessy, *Hypertension, Dietary Salt Intake, and the Role of the Thiazide-Sensitive Chloride Transporter NCCT.* Cardiovascular Therapeutics, 2011. **29**: p. 68-76.

4. N.I.C.E., *Hypertension*, in *Clinical management of primary hypertension in adults*, N.G.D.a.R. Unit, Editor. 2011: National Clinical Guideline Centre.

5. Mills, K.T., et al., *Global Disparities of Hypertension Prevalence and Control: A Systematic Analysis of Population-based Studies from 90 Countries.* Circulation, 2016. **134**(6): p. 441-450.

6. Glover, M. and K.M. O'Shaughnessy, *Molecular insights from dysregulation of the thiazide-sensitive WNK/SPAK/NCC pathway in the kidney: Gordon syndrome and thiazide-induced hyponatraemia.* Clin Exp Pharmacol Physiol, 2013. **40**(12): p. 876-84.

7. Felder, R.A., et al., *Diagnostic tools for hypertension and salt sensitivity testing.* Current Opinion in Nephrology and Hypertension, 2012. **22**: p. 65-76.

8. Association, A.H. *Know Your Risk Factors for High Blood Pressure*. 2017 [cited 2019 21.01.]; Available from: https://www.heart.org/en/health-topics/high-blood-pressure/why-high-blood-pressure-is-a-silent-killer/know-your-risk-factors-for-high-blood-pressure.

9. Mayan, H., et al., *Familial Hyperkalaemia and Hypertension: Pathogenic Insights Based on Lithium Clearance.* Journal Clinical Endocrinology Metabolism, 2009. **8**: p. 3010-3016.

10. Garovic, V.D., A.A. Hilliard, and S.T. Turner, *Monogenic Forms of Low-Renin Hypertension.* Nature Clinical Practice Nephrology, 2006. **2**(11): p. 624-630.

11. Organisation, W.H., *A global brief on Hypertension*, W.H.O., Editor. 2013, W.H.O: Switzerland. p. 1-40.

12. NHS. *High blood pressure (hypertension)*. 2018 [cited 2019; Available from: https://www.nhsinform.scot/illnesses-and-conditions/heart-and-blood-vessels/conditions/high-blood-pressure-hypertension#symptoms-of-high-blood-pressure.

13. Bloomfield, D.A. and A. Park, *Decoding white coat hypertension.* World J Clin Cases, 2017. **5**(3): p. 82-92.

14. Corbetta, S., et al., *Urinary exosomes in the diagnosis of Gitelman and Bartter syndromes.* Nephrology Dialysis Transplantations, 2014. **30**: p. 621-630.

15. Hamilton, K.L. and D.C. Devor, *Ion Channels and Transporters of Epithelia in Health and Disease*, ed. A.P. Society. 2016, New York: Springer.

16. Verouti, S.N., et al., *Regulation of blood pressure and renal function by NCC and ENaC: lessons from genetically engineered mice.* Current Opinion in Pharmacology, 2015. **21**: p. 60-72.

17. Gambo, G., *The thiazide-sensitive Na+Cl- cotransporter: molecular biology, functional properties, and regulation by WNKs.* American Journal of Physiology, 2009. **4**: p. 838-848.

18. Mayan, H., et al., *Increased urinary Na-Cl cotransporter protein in familial hyperkalaemia and hypertension.* Nephrology Dialysis Transplantations, 2008. **23**: p. 492-496.

19. Roy, A., et al., *Generation of WNK1 knockout cell lines by CRISPR/Cas-mediated genome editing.* American Journal of Renal Physiology, 2014. **308**: p. 366-376.

20. Tutakhel, O.A.Z., et al., *Alternative splice variant of the thiazide-sensitive NaCl cotransporter: A novel player in renal salt handling.* American Journal of Renal Physiology, 2015. **310**: p. 204-216.

21. Leviel, F., et al., *The Na+-dependent chloride-bicarbonate exchanger SCL4A8 mediates an electroneutral Na+ reabsorption process in the renal cortical collecting ducts of mice.* The Journal of Clinical Investigation, 2010. **5**: p. 1627-1635.

22. Fremont, O.T. and J.C.M. Chan, *Understanding Bartter syndrome and Gitelman syndrome.* World Journal of Pediatrics, 2012. **8**(1): p. 25-30.

23. Seys, E., et al., *Clinical and Genetic Spectrum of Bartter Syndrome Type 3.* J Am Soc Nephrol, 2017. **28**: p. 2540–2552.

24. Laghmani, K., et al., *Polyhydramnios, Transient Antenatal Bartter's Syndrome, and MAGED2 Mutations.* N Engl J Med, 2016. **374**: p. 1853-1863.

25. Paver, W.K. and G.J. Pauline, *HYPERTENSION AND HYPERPOTASSAEMIA WITHOUT RENAL DISEASE IN A YOUNG MALE.* The Medical Journal of Australia, 1964. **2**: p. 305-306.

26. O'Shaughnessy, K., *Gordon Syndrome: a continuing story.* Pediatric Nephrology, 2014. **11**: p. 1903-1908.

27. Gordon, R.D., *Syndrome of Hypertension and Hyperkalemia with Normal Glomerular Filtration Rate.* Hypertension, 1986. **8**: p. 93-102.

28. Sohara, E. and S. Uchida, *Kelch-like 3/Cullin 3 ubiquitin ligase complex and WNK signalling in salt-sensitive hypertension and electrolyte disorder.* Nephrology Dialysis Transplantations, 2015. **0**: p. 1-8.

29. Luft, F.C., F. Volhard, and K.-B.a.M. Delbrück, *Mendelian forms of Human Hypertension and Mechanisms of Disease.* Clinical Medicine & Research, 2003. **1**(4): p. 291-300.

30. BILGINTURAN, N., et al., *Hereditary Brachydactyly Associated with Hypertension.* Journal of Medical Genetics, 1973. **10**: p. 253-259.

31. Toka, O., et al., *Clinical Effects of Phosphodiesterase 3A Mutations in Inherited Hypertension With Brachydactyly.* Hypertension, 2015. **66**: p. 800-808.

32. Piala, A.T., et al., *Chloride sensing by WNK1 kinase involves inhibition of autophosphorylation.* Science Signalling, 2014. **7**: p. 1-21.

33. Rodan, A.R. and C.-L. Huang, *An Emerging Role for SPAK in NCC, NKCC, and Blood Pressure Regulation.* Journal American society of Nephrology, 2010. **21**: p. 1813-1815.

34. Chavez-Canales, M., et al., *WNK-SPAK-NCC Cascade Revisited: WNK1 Stimulates the Activity of the Na-Cl Cotransporter via SPAK, an Effect Antagonized by WNK4.* Hypertension, 2014. **64**: p. 1047-1053.

35. Liu, F., et al., *Common Variation in With No-Lysine Kinase 1 (WNK1) and Blood Pressure Responses to Dietary Sodium or Potassium Interventions.* Circulation Journal, 2013. **77**: p. 169-174.

36. Hadchouel, J., et al., *Familial Hyperkalemic Hypertension.* American Society of Nephrology, 2006. **17**: p. 208-217.

37. Verouti, S.N., et al., *Regulation of blood pressure and renal function by NCC and ENaC: lessons from genetically engineered mice.* Current Opinion in Pharmacology, 2015. **32**: p. 60-72.

38. Liu, F., et al., *Lack of Family-Based Association between Common Variations in WNK1 and Blood Pressure Level.* Medical Science Monitor, 2014. **20**: p. 1958-1962.

39. Glover, M. and K.M. O'Shaughnessy, *Molecular insights from dysregulation of the thiazide-sensitive WNK/SPAK/NCC pathway in the kidney: Gordon syndrome and thiazide-induced hyponatraemia.* Clinical and Experimental Pharmacology and Physiology, 2013. **40**: p. 876-884.

40. Glover, M., et al., *Detection of mutations in KLHL3 and CUL3 in families with FHHt (familial hyperkalaemic hypertension or Gordon's syndrome).* Clinical Science, 2014. **126**: p. 721-726.

41. Lai, L., et al., *Dietary salt modulates the sodium chloride cotransporter expression likely through an aldosterone-mediated WNK4-ERK1/2 signaling pathway.* Pflugers Arch, 2012. **463**(3): p. 477-85.

42. Rafiqi, F.H., et al., *Role of the WNK-activated SPAK kinase in regulating blood pressure.* EMBO Molecular Medicine, 2009. **2**: p. 63-75.

43. Rojas-Vega, L. and G. Gamba, *Mini-review: regulation of the renal NaCl cotransporter by hormones.* Am J Physiol Renal Physiol, 2016. **310**(1): p. F10-4.

44. Chavez-Canales, M., et al., *Insulin increases the functional activity of the renal NaCl cotransporter.* J Hypertens, 2013. **31**(2): p. 303-11.

45. Nishida, H., et al., *Phosphatidylinositol 3-kinase/Akt signaling pathway activates the WNK-OSR1/SPAK-NCC phosphorylation cascade in hyperinsulinemic db/db mice.* Hypertension, 2012. **60**(4): p. 981-90.

46. Sohara, E., et al., *Acute insulin stimulation induces phosphorylation of the Na-Cl cotransporter in cultured distal mpkDCT cells and mouse kidney.* PLoS One, 2011. **6**(8): p. e24277.

47. Kostakis, I.D., et al., *A Case Report of Gordon's Syndrome in a 20-Year-Old Male with Free Medical Family History.* Hellenic J Cardiol, 2013. **54**: p. 64-68.

48. Schumacher, F.-R., et al., *Characterisation of the Cullin-3 mutation that causes a severe form of familial hypertension and hyperkalaemia.* EMBO Molecular Medicine, 2015. **7**: p. 1285-1306.

49. Shibata, S., et al., *Kelch-like 3 and Cullin 3 regulate electrolyte homeostasis via ubiquitination and degradation of WNK4.* Proceedings of the National Academy of Sciences, 2013. **110**: p. 7838-7843.

50. NICE. *Hyponatraemia: Management of hyponatraemia*. 2015; Available from: https://cks.nice.org.uk/hyponatraemia#!scenario.

51. Wijst, J.v.d., et al., *Effects of a high sodium-low potassium diet on renal calcium, magnesium, and phosphate handling.* American Journal of Renal Physiology, 2018. **315**(1): p. F110-F122.

52. Chiga, M., et al., *Dietary salt regulates the phosphorylation of OSR1/SPAK kinases and the sodium chloride cotransporter through aldosterone.* Kidney Int, 2008. **74**: p. 1403-1409.

53. Haddy, F.J., P.M. Vanhoutte, and M. Feletou, *Role of potassium in regulating blood flow and blood pressure.* Am J Physiol Regul Integr Comp Physiol, 2006. **290**(3): p. R546-52.

54. Veiras, L.C., et al., *Potassium Supplementation Prevents Sodium Chloride Cotransporter Stimulation During Angiotensin II Hypertension.* Hypertension, 2016. **68**(4): p. 904-12.

55. Webb, T.N., et al., *Cell-specific regulation of L-WNK1 by dietary K^+.* American Journal of Renal Physiology, 2015. **310**: p. 15-26.

56. Manger, W., et al., *Protective effects of dietary potassium chloride on hemodynamics of Dahl salt-sensitive rats in response to chronic administration of sodium chloride.* Journal of Hypertension, 2003. **21**(12): p. 2305-2313.

57. Louis, W., R. Tabei, and S. Spector, *Effects of sodium intake on inherited hypertension in the rat.* Lancet, 1971. **2**(7737): p. 1283-1286.

58. Svetkey, L., et al., *Double-blind, placebo-controlled trial of potassium chloride in the treatment of mild hypertension.* Hypertension, 1987. **9**(5): p. 444-450.

59. MacGregor, G., et al., *Moderate potassium supplementation in essential hypertension.* Lancet, 1982. **2**(8298): p. 565-570.

60. Moore, T.J., et al., *DASH (Dieatary Approaches to Stop Hypertension) Diet is Effective Treatment for Stage 1 Isolated Systolic Hypertension.* Hypertension, 2001. **38**: p. 155-158.

61. Steinberg, D., G.G. Bennett, and L. Svetkey, *The DASH Diet, 20 Years Later.* Jama, 2017. **317**(15): p. 1529.

62. McCallum, L., S. Lip, and S. Padmanabhan, *The hidden hand of chloride in hypertension.* Pflugers Arch, 2015. **467**(3): p. 595-603.

63. Shoda, W., et al., *Calcineurin inhibitors block sodium-chloride cotransporter dephosphorylation in response to high potassium intake.* Kidney International, 2017. **91**: p. 402-411.

64. Hoorn, E., et al., *The calcineurin inhibitor tacrolimus activates the renal sodium chloride cotransporter to cause hypertension.* Nature Medicine, 2011. **17**(10): p. 1304-1309.

65. N.I.C.E. *Hypertension: clincial management of primary hypertension in adults. Clinical guideline* 2011 [cited 2014 May 1st]; CG127:[Available from: https://www.nice.org.uk/guidance/cg127.

66. Salvetti, A., et al., *Angiotensin-converting enzyme inhibitors in hypertension: a review.* Int J Clin Pharmacol Res, 1985. **5**(6): p. 429-438.

67. FH, M., et al., *Angiotensin-Converting Enzyme Inhibitors in Hypertension: To Use or Not to Use?* J Am Coll Cardiol, 2018. **71**(13): p. 1474-1482.

68. Muntwyler, J. and F. Follath, *Calcium Channel Blockers in Treatment of Hypertension.* Progress in Cardiovascular Diseases. **44**(3): p. 207-216.

69. Moser, M., *Fifty Years of Thiazide Diuretic Therapy for Hypertension.* American Medical Association, 2009. **169**(20): p. 1851-1856.

70. Palmer, B. and D. Clegg, *Renal Considerations in the Treatment of Hypertension.* American Journal of Hypertension, 2018. **31**(4): p. 394-401.

71. Hwang, K.S. and G.-H. Kim, *Thiazide-Induced Hyponatremia.* The Korean Society of Electrolyte Metabolism, 2010. **17**: p. 51-57.

72. Barber, J., et al., *A systematic review and meta-analysis of thiazide-induced hyponatraemia: time to reconsider electrolyte monitoring regiments after thiazide initiation?* British Journal of Clinical Pharmacology, 2014. **79**: p. 566-577.

73. NICE. *Hyponatraemia*. 2015 [cited 2019 21.01]; Available from: https://cks.nice.org.uk/hyponatraemia#!topicsummary.

74. Friedman, E., et al., *Thiazide-Induced Hyponatremia: Reproducility by Single Dose Rechallenge and an Analysis of Pathogenesis.* American College of Physicians, 1989. **110**: p. 24-30.

75. Glover, M. and J. Clayton, *Thiazide-induced hyponatraemia: epidemiology and clues to pathogenesis.* Cardiovasc Ther, 2012. **30**(5): p. e219-26.

76. Assadi, F., *Hyponatremia: a problem-solving approach to clinical cases.* J Nephrol, 2012. **25**(4): p. 473-80.

77. Ware, J.S., et al., *Phenotypic and pharmacogenetic evaluation of patients with thiazide-induced hyponatremia.* J Clin Invest, 2017. **127**(9): p. 3367-3374.

78. Assadi, F., *Hyponatremia: a problem-solving approach to clinical cases.* Journal of Nephrology, 2012. **4**: p. 473-480.

79. Frenkel, N.J., et al., *Thiazide-induced hyponatraemia is associated with increased water intake and impaired urea-mediated water excretion at low plasma antidiuretic hormone and urine aquaporin-2.* Journal of Hypertension, 2015. **33**: p. 627-633.

80. Pillai, B.P., A.G. Unnikrishnan, and P.V. Pavithran, *Syndrome of inappropriate antidiuretic hormone secretion: Revisiting a classical endocrine disorder.* Indian Journal of Endocrinology Metabolism, 2011. **15**: p. S208-S215.

81. Nadal, J., et al., *Clinical and Molecular Features of Thiazide-Induced Hyponatremia.* Current Hypertension, 2018. **20**(31): p. 1-7.

82. Bartter, F.C. and W.B. Schwartz, *The syndrome of inappropriate secretion of antidiuretic hormone.* The American Journal of Medicine, 1967. **42**(5): p. 790-806.

83. Gross, P., *Clinical management of SIADH.* Ther Adv Endocrinol Metab, 2012. **3**(2): p. 61-73.

84. Adrogué, H.J., *HYPONATREMIA IN HEART FAILURE.* Methodist Debakey Cardiovasc Journal, 2017. **13**(1): p. 40.

85. Abraham, W.T., *Managing Hypnatremia in Heart Failure.* US Cardiology, 2008. **5**(1): p. 57-60.

86. Xu, W.C., et al., *Urinary Extracellular Vesicle: A Potential Source of Early Diagnostic and Therapeutic Biomarker in Diabetic Kidney Disease.* Chin Med J (Engl), 2018. **131**(11): p. 1357-1364.

87. Wang, D. and W. Sun, *Urinary extracellular microvesicles: isolation methods and prospects for urinary proteome.* Proteomics, 2014. **14**(16): p. 1922-32.

88. Cocucci, E. and J. Meldolesi, *Ectosomes and exosomes: shedding the confusion between extracellular vesicles.* Trends Cell Biol, 2015. **25**(6): p. 364-72.

89. Moreno-Gonzalo, O., CarolinaVillarroya-Beltri, and F. Sánchez-Madrid, *Post-translational modifications of exosomal proteins.* Frontiers in Immunology, 2014. **5**(383): p. 1-7.

90. Morrison, E., *Development of circulatory microRNAs as markers of organ injury and mediators of interorgan signalling*, in *College of Medicine and Veterinary Medicine*. 2018, University of Edinburgh. p. 268.

91. Zha, Q.B., et al., *Extracellular vesicles: An overview of biogenesis, function, and role in breast cancer.* Tumour Biology, 2017: p. 1-7.

92. Akers, J.C., et al., *Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies.* Journal of Neurooncology, 2013. **112**: p. 1-11.

93. Vader, P., X.O. Breakefield, and M.J.A. Wood, *Extracellular vesicles: emerging targets for cancer therapy.* Trends in Molecular Medicine, 2014. **20**(7): p. 395-393.

94. Moon, P.-G., et al., *Urinary Exosomes and Proteomics.* Mass Spectrometry Reviews, 2009. **30**: p. 1185-1202.

95. Esteva-Font, C., et al., *Are sodium Transporters in Urinary Exosomes Reliable Markers of Tubular Sodium Reabsorption in Hypertensive Patients?* Nephron Physiology, 2010. **114**: p. 25-34.

96. Katsuda, T., et al., *The Therapeutic potential of mesenchymal stem cell-deprived extracellular vesicles.* Proteomics, 2013. **13**: p. 1637-1653.

97. fang, D.Y., et al., *Exosomes and the kidney: blaming the messenger.* Nephrology, 2013. **18**: p. 1-10.

98. Lubbe, N.v.d., et al., *The Phosphorylated Sodium Chloride Cotransporter in Urinary Exosomes Is Superior to Prostasin as a Marker for Aldosteronism.* Hypertension, 2012. **60**(741-748).

99. Corrado, C., et al., *Exosomes as intercellular signaling organelles involved in health and disease: basic science and clinical applications.* Int J Mol Sci, 2013. **14**(3): p. 5338-66.

100. Biancone, L., et al., *Therapeutic potential of mesenchymal stem cell-derived microvesicles.* Nephrology Dialysis Transplantations, 2012. **27**: p. 3037-3042.

101. Barasch, J., et al., *Urine: Waste product or biologically active tissue?* Neurourology and Urodynamics, 2018: p. 1-7.

102. Heinemann, M.L., et al., *Benchtop isolation and characterisation of functional exosomes by sequential filtration.* Journal of Chromatography, 2014. **1371**: p. 125-135.

103. Balkom, B.W.M.v., et al., *Exosomes and the kidney: prospects for diagnosis and therapy of renal disease.* International Society of Nephrology, 2011. **80**: p. 1138-1145.

104. Cheruvanky, A., et al., *Rapid Isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator.* American Journal of Renal Physiology, 2007. **292**: p. 1657-1661.

105. Nilsson, J., et al., *Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer.* British Journal of Cancer, 2009. **100**(10): p. 1603-1607.

106. Bachmann, S., et al., *Renal effects of Tamm-Horsfall protein (uromodulin) deficiency in mice.* American Journal of Renal Physiology, 2004. **288**: p. 559-567.

107. Padmanabhan, S., et al., *Uromodulin, an Emerging Novel Pathway for Blood Pressue Regulation and Hypertension.* Hypertension, 2014. **64**: p. 918-923.

108. Fernandez-Llama, P., et al., *Tamm-Horsfall protein and urinary exosome isolation.* Kidney International, 2010. **77**: p. 736-742.

109. Glover, M. and J. Clayton, *Thiazide-Induced Hyponatraemia: Epidemiology and Clues to Pathogenesis.* Cardiovascular Therapeutics, 2011. **30**: p. 219-226.

110. Wang, D. and W. Sun, *Urinary extracellular microvesicles: Isolation methods and prospects for urinary proteome.* Proteomics, 2014. **14**: p. 1922-1932.

111. Pearce, D., et al., *Collecting duct principal cell transport processes and their regulation.* Clin J Am Soc Nephrol, 2015. **10**(1): p. 135-46.

112. Radin, M.J., et al., *Aquaporin-2 regulation in health and disease.* Veterinary Clinical Pathology, 2012. **41**: p. 455-470.

113. Pisitkun, T., R.F. Shen, and M.A. Knepper, *Identification and proteomic profiling of exosomes in human urine.* Proc Natl Acad Sci U S A, 2004. **101**(36): p. 13368-73.

114. Chatellard-Causse, C., et al., *Alix (ALG-2-interacting Protein X), a Protein Involved in Apoptosis, Binds to Endophilins and Induces Cytoplasmic Vacuolization.* Journal of Biological Chemistry, 2002. **277**(32): p. 29108-29115.

115. Hurley, J.H. and G. Odorizzi, *Get on the exosome bus with ALIX.* Nature Cell Biology, 2012. **14**(7): p. 654-655.

116. Wolley, M.J., et al., *In Primary Aldosteronism, Mineralocorticoids Influence Exosomal Sodium-Chloride Cotransporter Abundance.* J Am Soc Nephrol, 2017. **28**(1): p. 56-63.

117. Channavajjhala, S.K., et al., *Urinary Extracellular Vesicle Protein Profiling and Endogenous Lithium Clearance Support Excessive Renal Sodium Wasting and Water Reabsorption in Thiazide-Induced Hyponatremia.* Kidney Int, 2019. **4**(1): p. 139-147.

118. Miyazawa, Y., et al., *AQP2 in human urine is predominantly localized to exosomes with preserved water channel activities.* Clincial and Experimental Nephrology, 2018. **1**: p. 1-7.

119. Willms, E., et al., *Cells release subpopulations of exosomes with distinct molecular and biological properties.* Sci Rep, 2016. **6**: p. 22519.

120. Mignot, G., et al., *Prospects for exosomes in immunotherapy of cancer.* Journal of Cell and Molecular Medicine, 2006. **10**(2): p. 376-388.

121. Moon, P.G., et al., *Proteomic analysis of urinary exosomes from patients of early IgA nephropathy and thin basement membrane nephropathy.* Proteomics, 2011. **11**(12): p. 2459-75.

122. Bussolati, B., et al., *Human renal cancer stem cells.* Cancer Lett, 2013. **338**(1): p. 141-6.

123. Grange, C., et al., *Microvesicles released from human renal cancer stem cells stimulate angiogenesis and formation of lung premetastatic niche.* Cancer Res, 2011. **71**(15): p. 5346-56.

124. Toro, J.D., et al., *Emerging roles of exosomes in normal and pathological conditions: new insights for diagnosis and therapeutic applications.* Frontiers in Immunology, 2015. **6**(203): p. 1-12.

125. Taub, P.R., et al., *Role of biomarkers in the diagnosis and prognosis of acute kidney injury in patients with cardiorenal syndrome.* Expert Review of Cardiovascular Therapy, 2012. **10**: p. 657-667.

126. van Balkom, B.W.M., et al., *Exosomes and the kidney: prospects for diagnosis and therapy of renal diseases.* Kidney International, 2011. **80**(11): p. 1138-1145.

127. Coulter, B. *AMPure XP beads purification*. 2017; Available from: https://www.beckmancoulter.com.

128. Ilumina. *RNA library prepation and sequencing*. 2018 [cited 2019 18.01]; Available from: https://international.neb.com/applications/.

129. Trapnell, C., et al., *Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks.* nature Protocols, 2012. **7**(3): p. 562-578.

130. Hanukoglu, I. and A. Hanukoglu, *Epithelial sodium channel (ENaC) family: Phylogeny, structure-function, tissue distribution, and associated inherited diseases.* Gene, 2016. **579**(2): p. 95-132.

131. Thery, C., et al., *Isolation and Characterization of Exosomes from Cell Culture Supernatants and Biological Fluids.* Subcellular Fractionatio and Isolation of Organelles, 2006. **3**(22): p. 1-29.

132. Van Deun, J., et al., *The impact of disparate isolation methods for extracellular vesicles on downstream RNA profiling.* J Extracell Vesicles, 2014. **3**.

133. Wu, Y., W. Deng, and D.J. Klinke, 2nd, *Exosomes: improved methods to characterize their morphology, RNA content, and surface protein biomarkers.* Analyst, 2015. **140**(19): p. 6631-42.

134. Erdbrügger, U. and T.H. Le, *Extracellular Vesicles in Renal Diseases: More than Novel Biomarkers?* Journal American Society of Nephrology, 2016. **27**: p. 12-26.

135. Lawson, C., et al., *Microvesicles and exosomes: new players in metabolic and cardiovascular disease.* Journal of Endocrinology, 2016. **228**(2): p. R57-R71.

136. Schrier, R.W., *Vasopressin and Aquaporin 2 (AQP2) in Clinical Disorders of Water Homeostasis.* Semin Nephrol., 2008. **28**(3): p. 289-296.

137. Higashijima, Y., et al., *Excretion of urinary exosomal AQP2 in rats is regulated by vasopressin and urinary pH.* American Journal of Renal Physiology, 2013. **305**: p. F1412-F1421.

138. Odorizzi, G., *The multiple personalities of Alix.* Journal of Cell Science, 2006. **119**(5): p. 3025-3032.

139. Baietti, M.F., et al., *Syndecan–syntenin–ALIX regulates the biogenesis of exosomes.* Nature Cell Biology, 2012. **14**(7): p. 677-685.

140. Cheruvanky, A., et al., *Rapid isolation of urinary exosomal biomarkers using a nanomembrane ultrafiltration concentrator.* Am J Physiol Renal Physiol, 2007. **292**(5): p. F1657-61.

141. Andaloussi, S.E., et al., *Extracellular vesicles: biology and emerging therapeutic opportunities.* NATURE REVIEWS, 2013. **12**: p. 347-357.

142. G., M., et al., *Prospects for exosomes in immunotherapy of cancer.* Journal of Cellular and Molecular Medicine, 2006. **10**(2): p. 376-388.

143. Nilsson, J., et al., *Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer.* Br J Cancer, 2009. **100**(10): p. 1603-7.

144. Li, J., et al., *Identification and Characterization of 293T Cell-Derived Exosomes by Profiling the Protein, mRNA and MicroRNA Components.* PLoS ONE, 2016. **11**(9): p. e0163043.

145. Technologies, L., *Qtracker Cell Labeling Kits*, M. Probes, Editor. 2013. p. 1-9.

146. van der Lubbe, N., et al., *The phosphorylated sodium chloride cotransporter in urinary exosomes is superior to prostasin as a marker for aldosteronism.* Hypertension, 2012. **60**(3): p. 741-8.

147. Pacheco-Alvarez, D., et al., *The Na+:Cl- Cotransporter Is Activated and Phosphorylated at the Amino=terminal Domain upon Intracellular Chloride Depletion.* The Journal of Biological Chemistry, 2006. **281**(39): p. 28755-28763.

148. MA Knepper and H. Brooks, *Regulation of the sodium transporters NHE3, NKCC2 and NCC in the kidney.* Current Opinion in Nephrology and Hypertension, 2001. **10**(5): p. 655-659.

149. Palmer, L.G. and J. Schnermann, *Integrated Control of Na Transport along the Nephron.* Clinical Journal American Soceity of Nephrology, 2015. **10**: p. 676-687.

150. Guo, B.B., S.A. Bellingham, and A.F. Hill, *Stimulating the Release of Exosomes Increases the Intercellular Transfer of Prions.* The Journal of Biological Chemistry, 2016. **291**(10): p. 5128-5137.

151. Emam, S.E., et al., *A Novel Strategy to Increase the Yield of Exosomes (Extracellular Vesicles) for an Expansion of Basic Research.* Biol. Pharm. Bull, 2018. **41**(5): p. 733-742.

152. Jella, K.K., et al., *Exosomes, Their Biogenesis and Role in Inter-Cellular Communication, Tumor Microenvironment and Cancer Immunotherapy.* Vaccines, 2018. **6**(69): p. 1-15.

153. Behbahani, G.D., et al., *The role of exosomes contents on genetic and epigenetic alterations of recipient cancer cells.* Iranian Journal of Basic Medical Sciences, 2016. **19**(10): p. 1031-1039.

154. Terker, A.S., et al., *Unique chloride-sensing properties of WNK4 permit the distal nephron to modulate potassium homeostasis.* Kidney Int, 2016. **89**(1): p. 127-34.

155. Terker, A.S., et al., *Potassium modulates electrolyte balance and blood pressure through effects on distal cell voltage and chloride.* Cell Metab, 2015. **21**(1): p. 39-50.

156. Glover, M., A.M. Zuber, and K.M. O'Shaughnessy, *Hypertension, Dietary Salt Intake, and the Role of the Thiazide-Sensitive Sodium Chloride Transporter NCCT.* Cardiovascular Therapeutics, 2011. **29**(1): p. 68-76.

157. Ko, B., et al., *Mechanisms of angiotensin II stimulation of NCC are time-dependent in mDCT15 cells.* Am J Physiol Renal Physiol, 2015. **308**: p. F720-F727.

158. San-Cristobala, P., et al., *Angiotensin II signaling increases activity of the renal Na-Cl cotransporter through a WNK4-SPAK-dependent pathway.* PNAS, 2009. **106**(11): p. 4384-4389.

159. Lucko, A.M., et al., *Percentage of ingested sodium excreted in 24 - hour urine collections: A systematic review and meta - analysis.* Journal of Clinical Hypertension, 2018. **20**: p. 1220-1229.

160. Birukov, A., et al., *Ultra-long–term human salt balance studies reveal interrelations between sodium, potassium, and chloride intake and excretion.* Am J Clin Nutr, 2016. **104**(49-57).

161. Penton, D., et al., *Extracellular K+ rapidly controls NaCl cotransporter phosphorylation in the native distal convoluted tubule by Cl- - dependent and independent mechanisms.* J Physiol, 2016. **594**(21): p. 6319-6331.

162. Gamba, G., *The thiazide-sensitive Na+-Cl- cotransporter: molecular biology, functional properties, and regulation by WNKs.* AJP: Renal Physiology, 2009. **297**(4): p. F838-F848.

163. Wade, J.B., et al., *SPAK-mediated NCC regulation in response to low-K diet.* American Journal of Renal Physiology, 2015. **308**: p. F923-931.

164. Murthy, M., T. Kurz, and K.M. O'Shaughnessy, *ROMK expression remains unaltered in a mouse model of familial hyperkalemic hypertension caused by the CUL3Delta403-459 mutation.* Physiol Rep, 2016. **4**(13).

165. Frindt, G. and L.G. Palmer, *Effects of dietary K on cell-surface expression of renal ion channels and transporters.* American Journal of Renal Physiology, 2010. **299**(4): p. F890-F897.

166. Rengarajan, S., et al., *Increasing plasma [K+] by intravenous potassium infusion reduces NCC phosphorylation and drives kaliuresis and natriuresis.* American Journal of Renal Physiology, 2014. **306**(9): p. F1059-F1068.

167. Sorensen, M.V., et al., *Rapid dephosphorylation of the renal sodium chloride cotransporter in response to oral potassium intake in mice.* Kidney Int, 2013. **83**: p. 811-824.

168. Ishizawa, K., et al., *Potassium depletion stimulates Na-Cl cotransporter via phosphorylation and inactivation of the ubiquitin ligase Kelch-like 3.* Biochem Biophys Res Commun, 2016. **480**(4): p. 745-751.

169. Castañeda-Bueno, M., et al., *Modulation of NCC activity by low and high K+ intake: insights into the signaling pathways involved.* American Journal of Renal Physiology, 2014. **306**(12): p. F1507-F1519.

170. WHITESCARVER, S.A., et al., *Effect of Dietary Chloride on Salt-Sensitive and Renin-Dependent Hypertension.* Hypertension, 1986. **8**: p. 56-61.

171. Kotchen, T.A., et al., *Effect of chloride on renin and blood pressure responses to sodium chloride.* Annual International Medicine, 1983. **98**(5): p. 817-822.

172. Boegehold, M.A. and T.A. Kotchen, *Importance of dietary chloride for salt sensitivity of blood pressure.* Hypertension, 1991. **17**(1): p. 158-161.

173. Berger, S.S. and M.H. Fineberg, *The Effect if Sodium Chloride on Hypertension.* Arch Intern Med, 1929. **44**(4): p. 531-542.

174. Alvarez, M.L., et al., *Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers.* Kidney Int, 2012. **82**(9): p. 1024-32.

175. Neal, C.S., et al., *Circulating microRNA expression is reduced in chronic kidney disease.* Nephrology Dialysis Transplantation, 2011. **26**(11): p. 3794-3802.

176. Wei, C., et al., *Circulating miRNAs as Potential Marker for Pulmonary Hypertension.* PLOS ONE, 2013. **8**(5): p. e64396.

177. Silva, G.M. and C. Vogel, *Quantifying gene expression: the importance of being subtle.* Molecular Systems Biology, 2016. **12**(885): p. 1-2.

178. ROSS, J., *mRNA Stability in Mammalian Cells.* MICROBIOLOGICAL REVIEWS,, 1995. **59**(3): p. 423-450.

179. Laurent, L.C., et al., *Meeting report: discussions and prelimary findings on extracellular RNA measurement methods from laboratories in the NIH Extracellular RNA Communications Consortium.* Journal of Extracellular Vesicles, 2015. **4**(1-15).

180. Tang, Y.-T., et al., *Comparison of isolation methods of exosomes and exosomal RNA from cell culture medium and serum.* INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE, 2017. **40**(3): p. 834-844.

181. Miranda, K.C., et al., *Nucleic acids within urinary exosomes/microvesicles are potential biomarkers for renal disease.* Kidney Int, 2010. **78**(2): p. 191-9.

182. Khurana, R., et al., *Identification of urinary exosomal non-coding RNA as nocel biomarkers in Chronic Kidney Disease.* RNA, 2017. **23**(2): p. 142-152.

183. Enderle, D., et al., *Characterization of RNA from Exosomes and Other Extracellular Vesicles Isolated by a Novel Spin Column-Based Method.* PLoS ONE, 2015. **10**(8): p. e0136133.

184. Sigma-Aldrich, *GenElute™ Mammalian Total RNA Miniprep Kit*. 2017, Sigma-Aldrich: St Louis. p. 16.

185. Miranda, K.C., et al., *Massively parallel sequencing of human urinary exosome/microvesicle RNA reveals a predominance of non-coding RNA.* PLoS One, 2014. **9**(5): p. e96094.

186. Lobb, R.J., et al., *Optimized exosome isolation protocol for cell culture supernatant and human plasma.* J Extracell Vesicles, 2015. **4**: p. 27031.

187. Huang, X., et al., *Characterization of human plasma-derived exosomal RNAs by deep sequencing.* BMC Genomics, 2013. **14**(319): p. 1-14.

188. Oosthuyzen, W., et al., *Quantification of human urinary exosomes by nanoparticle tracking analysis.* Journal of Physiology, 2013. **529**(23): p. 5833-5841.

189. Scientific, T. *How Taqman Works*. 2018 [cited 2019 07.02]; Available from: https://www.thermofisher.com/uk/en/home/life-science/pcr/real-time-pcr/real-time-pcr-learning-center/real-time-pcr-basics/how-taqman-assays-work.html.

190. Clayton, J.A., I.R.L. Jeune, and I.P. Hall, *Severe hyponatraemia in medical in-patients aetiology, assessment and outcome.* Q J Med, 2006. **99**: p. 505-511.

191. Huang, C.-C., et al., *Clinical and Genetic Factors Associated With Thiazide-Induced Hyponatremia.* Medicine, 2015. **94**(34): p. e1422.

192. Wang, T., *Renal outer medullary potassium channel knockout models reveal thick ascending limb function and dysfunction.* Clinical Experimental of Nephrology, 2012. **16**: p. 49-54.

193. Simon, D.B., et al., *Genetic heterogeneity of Bartter's syndrome revealed by mutations in the K+ channel, ROMK.* Nature Genetics, 1996. **14**: p. 152-156.

194. Farenholtz, J., et al., *Expression of Slc35f1 in the murine brain.* Nature, 2019. **https://doi.org/10.1007/s00441-019-03008-8**.

195. Rahnama, F., R. Toftgard, and P.G. Zaphiropoulos, *Distinct roles of PTCH2 splice variants in Hedgehog signaling.* Biochem Journal, 2004. **378**: p. 325-334.

196. Balkom, B.W.M.v., et al., *Quantitative and qualitative analysis of small RNAs in human endothelial cells and exosomes provides insights into localized RNA processing, degradation and sorting.* Journal of Extracellular Vesicles, 2015. **4**(1): p. 26760.

197. Desdín-Micó, G. and M. Mittelbrunn, *Role of exosomes in the protection of cellular homeostasis.* Cell Adhesion & Migration, 2017. **11**(2): p. 127-134.

198. Nakano, K., et al., *AJS1669, a novel small-molecule muscle glycogen synthase activator, improves glucose metabolism and reduces body fat mass in mice.* Internal Journal of Molecular Medicine, 2017. **39**(4): p. 841-850.

199. Knepper, M.A. and T. Pisitkun, *Exosomes in urine: Who would have thought…?* Kidney International, 2007. **72**(9): p. 1043-1045.

200. Pospichalova, V., et al., *Simplified protocol for flow cytometry analysis of fluorescently labeled exosomes and microvesicles using dedicated flow cytometer.* Journal of Extracellular Vesicles, 2015. **4**: p. 25530.

201. Morales-Kastresana, A., et al., *Labeling Extracellular Vesicles for Nanoscale Flow Cytometry.* Scientific Reports, 2017. **7**(1878): p. 1-10.

202. Mehdiani, A., et al., *An Innovative Method for Exosome Quantification and Size Measurement.* Journal of Visualized Experiments, 2015. **95**: p. e50974.

203. Wiklander, O.P.B., et al., *Systematic Methodological Evaluation of A Multiplex Bead-Based Flow Cytometry Assay for Detection of Extracellular Vesicle Surface Signatures.* Frontiers in Immunology, 2018. **9**(1326): p. 1-26.

204. Paddock, S.W., *Principles and Practices of Laser Scanning Confocal Microscopy.* Molecular Biotechnology, 2000. **16**: p. 127-149.

205. Gildea, J.J., et al., *Exosomal transfer from human renal proximal tubule cells to distal tubule and collecting duct cells.* Clinical Biochemistry, 2014. **47**: p. 89-94.

206. Jella, K.K., et al., *Exosomal GAPDH from Proximal Tubule Cells Regulate ENaC Activity.* PLoS ONE, 2016. **11**(11): p. 1-20.

207. García-Campos, M.A., J. Espinal-Enríquez, and E. Hernández-Lemus, *Pathway Analysis: State of the Art.* Frontiers in Physiology, 2015. **6**(383): p. 1-16.

9 Appendix

9.1 cDNA Nanodrop Pictographs

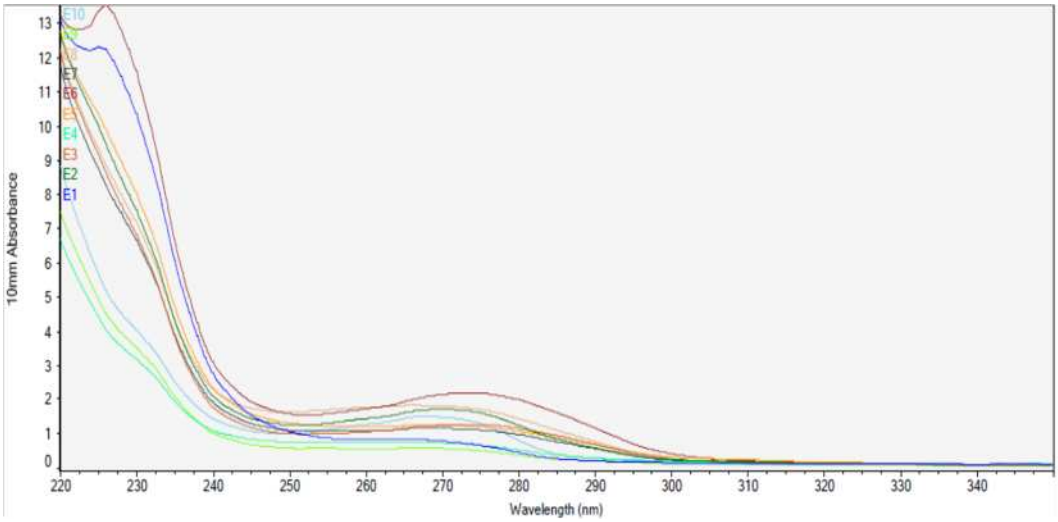


Figure 9-1 E1-E10 cDNA Nanodrop Pictographs

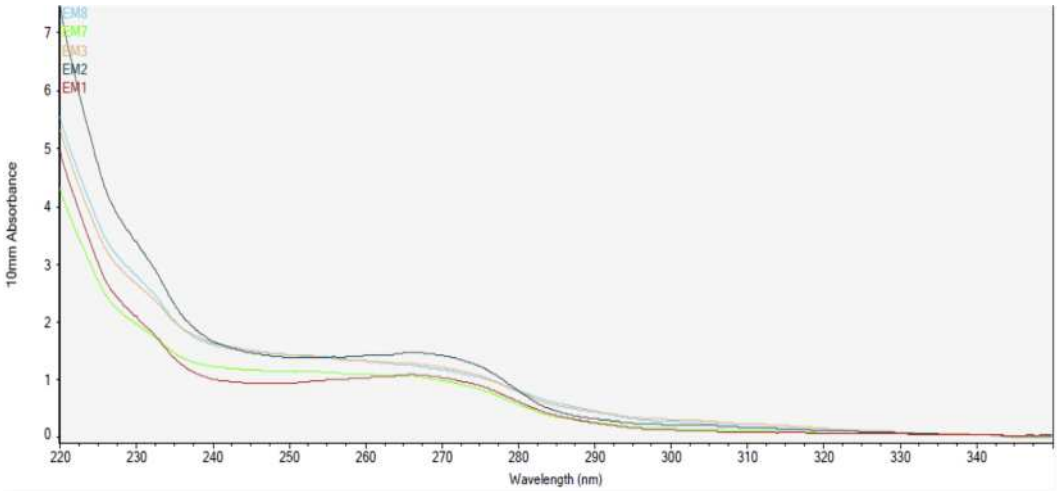


Figure 9-2 EM1-EM5 cDNA Nanodrop Pictograph

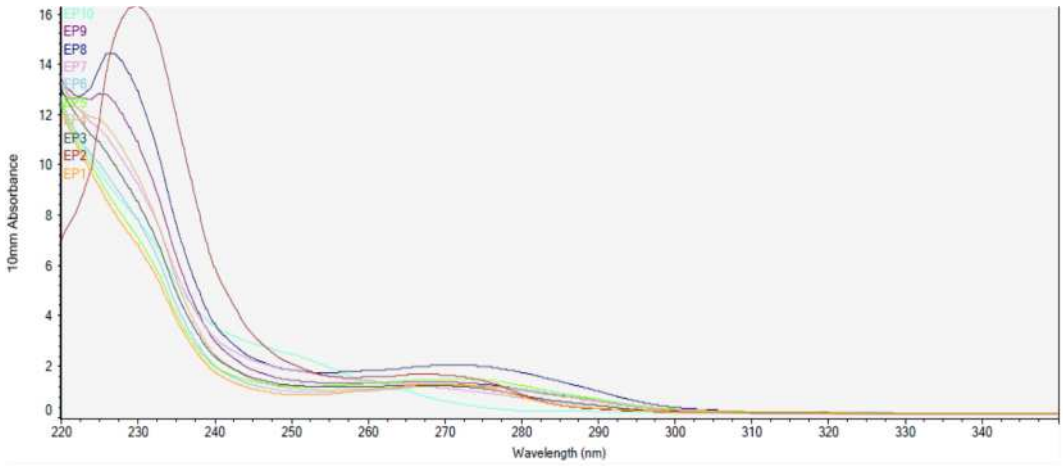


Figure 9-3 EP1-EP10 cDNA Nanodrop Pictograph

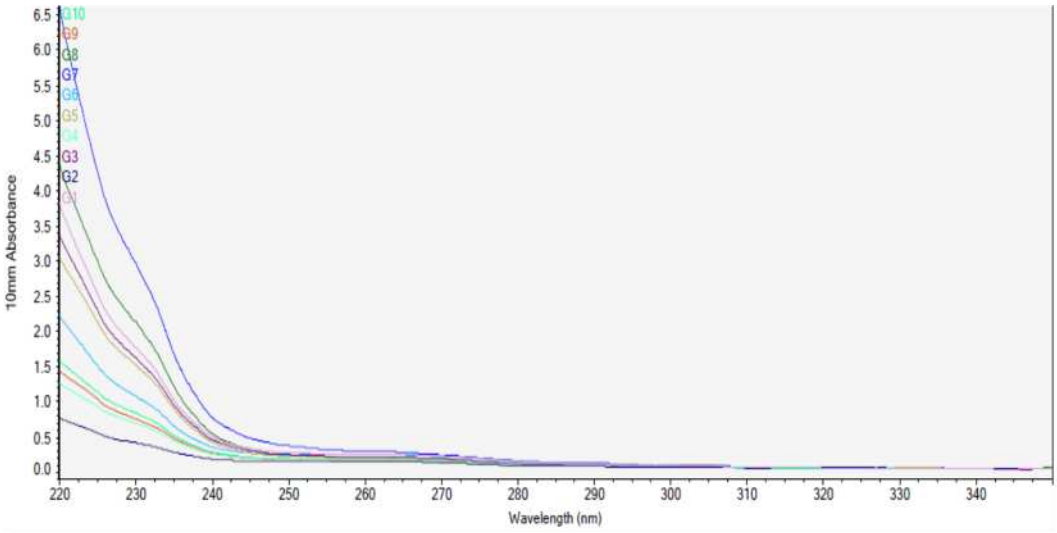


Figure 9-4 G1-G10 cDNA Nanodrop Pictograph

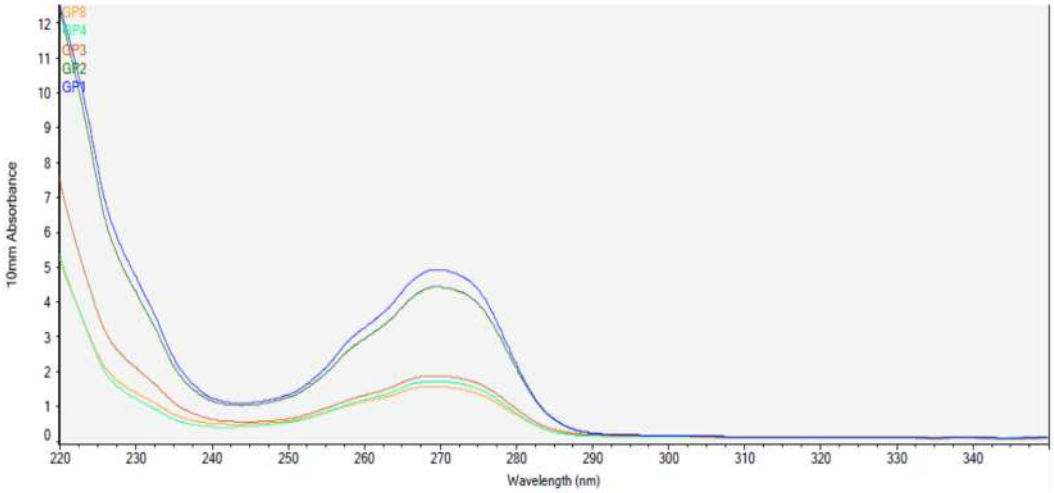


Figure 9-5 GP1-GP10 cDNA Nanodrop Pictograph

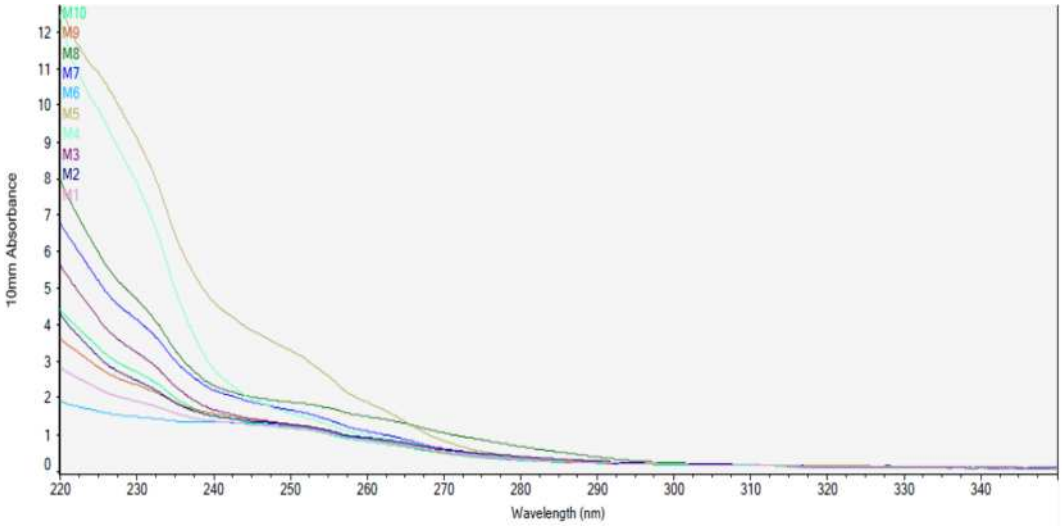


Figure 9-6 M1-M10 cDNA Nanodrop Pictograph

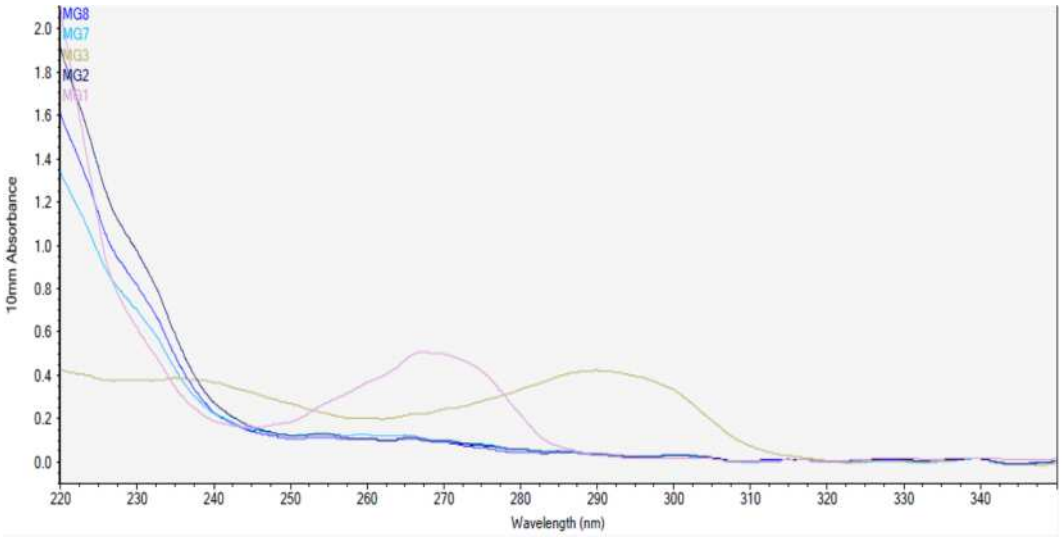


Figure 9-7 MG1-MG5 cDNA Nanodrop Pictograph

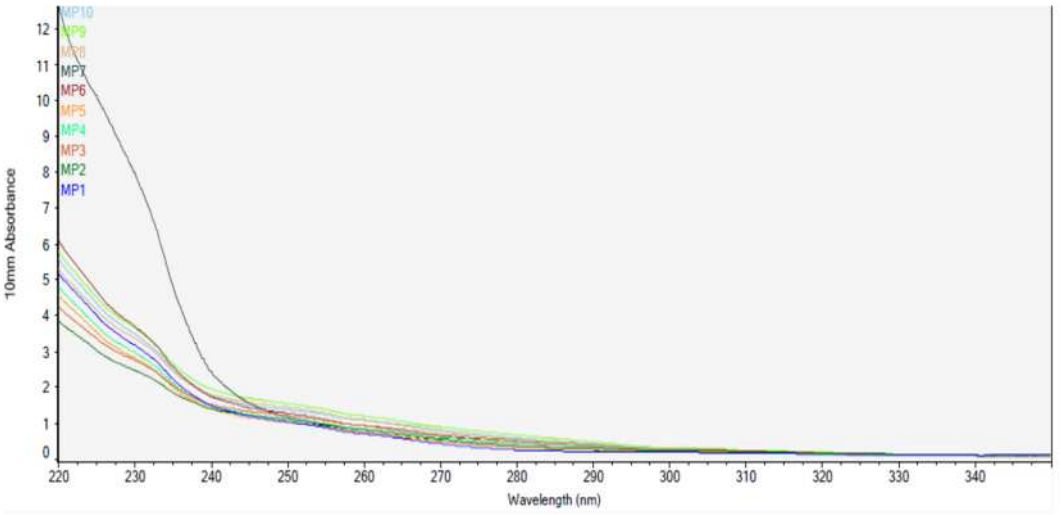


Figure 9-8 MP1-MP10 cDNA Nanodrop Pictograph

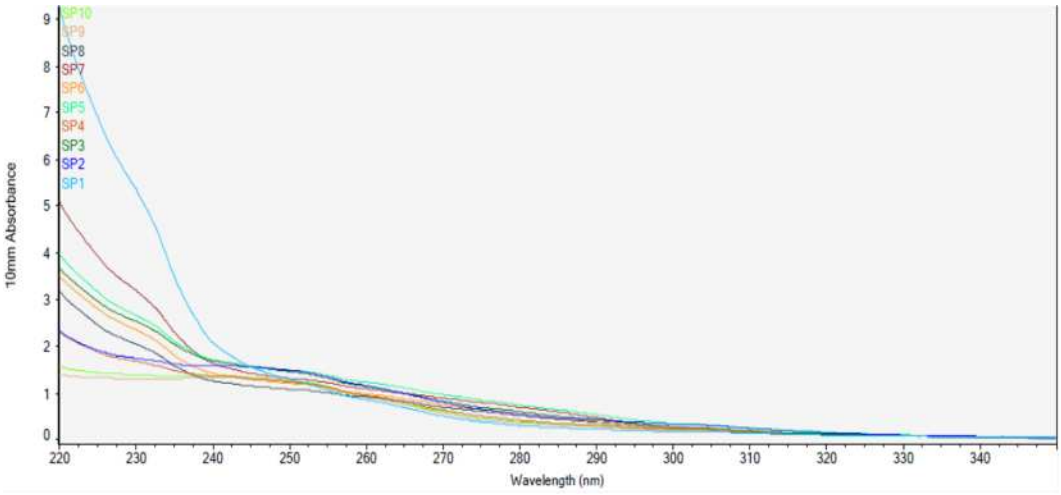


Figure 9-9 SP1-SP10 cDNA Nanodrop Pictograph

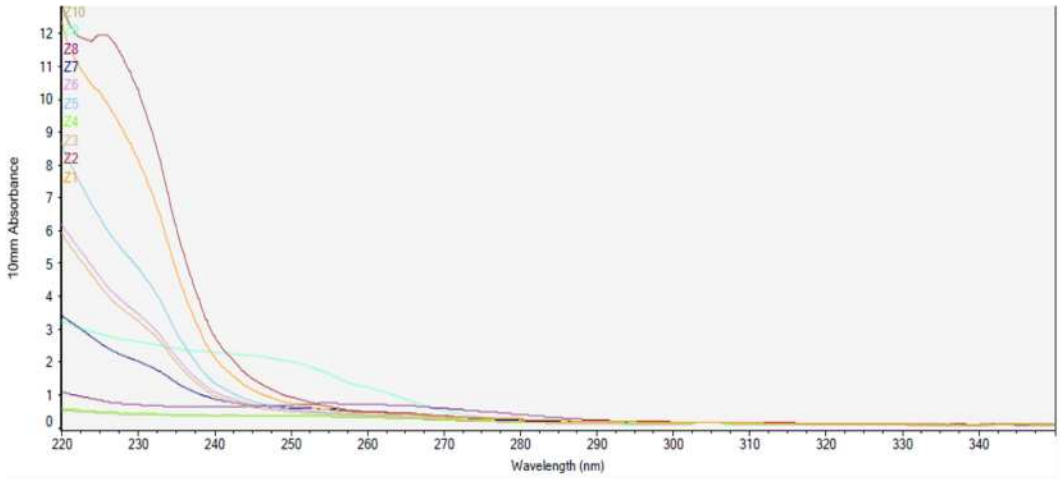


Figure 9-10 Z1-Z10 cDNA Nanodrop Pictograph

9.2 Published Paper

Current Hypertension Reports (2018) 20: 31
https://doi.org/10.1007/s11906-018-0826-6

BLOOD PRESSURE MONITORING AND MANAGEMENT (J COCKCROFT, SECTION EDITOR)



Clinical and Molecular Features of Thiazide-Induced Hyponatremia

Jodie Nadal[1] · Sarath K. Channavajjhala[1] · Wenjing Jia[1] · Jenny Clayton[2] · Ian P. Hall[1,3] · Mark Glover[1]

Published online: 10 April 2018
© The Author(s) 2018. This article is an open access publication

Abstract

Purpose of Review Hypertension affects more than 30% of the world's adult population and thiazide (and thiazide-like) diuretics are amongst the most widely used, effective, and least costly treatments available, with all-cause mortality benefits equivalent to angiotensin-converting enzyme inhibitors or calcium channel antagonists. A minority of patients develop thiazide-induced hyponatremia (TIH) and this is largely unpredictable at the point of thiazide prescription. In some cases, TIH can cause debilitating symptoms and require hospital admission. Although TIH affects only a minority of patients exposed to thiazides, the high prevalence of hypertension leads to TIH being the most common cause of drug-induced hyponatremia requiring hospital admission in the UK. This review examines current clinical and scientific understanding of TIH. Consideration is given to demographic associations, limitations of current electrolyte monitoring regimens, clinical presentation, the phenotype evident on routine clinical blood and urine tests as well as more extensive analyses of blood and urine in research settings, recent genetic associations with TIH, and thoughts on management of the condition.

Recent Findings Recent genetic and phenotyping analysis has suggested that prostaglandin E2 pathways in the collecting duct may have a role in the development of TIH in a subgroup of patients. Greater understanding of the molecular pathophysiology of TIH raises the prospect of pre-prescription TIH risk profiling and may offer novel insights into how TIH may be avoided, prevented and treated.

Summary The rising prevalence of hypertension and the widespread use of thiazides mean that further understanding of TIH will continue to be a pressing issue for patients, physicians, and scientists alike for the foreseeable future.

Keywords Hyponatremia · Thiazide-induced hyponatremia · TIH · Hypertension · Thiazide · Diuretics

Introduction to the Role of Thiazides in the Treatment of Hypertension

Hypertension is the most common modifiable cause of cardiovascular disease—the leading cause of mortality worldwide [1, 2]. At least 30% of the UK adult population have hypertension, and treatment consumes over a billion pounds per year in NHS drug costs alone [1, 3]. Whilst the treatment of hypertension is both evidence based and cost-effective [1], most patients require multiple antihypertensive agents, side effects are commonplace, and blood pressure control for many remains poor [3]. The current therapeutic limitations are perhaps not surprising since for most affected individuals we are still unable to define either the molecular mechanisms driving their hypertension or those leading to adverse effects from antihypertensive treatment.

The association of hypertension with salt is intriguing however; sodium chloride intake correlates with the prevalence of hypertension even in disparate populations [4], and sodium restriction lowers blood pressure, especially in those already hypertensive [5]. The physiological effects of salt on blood pressure are not a simple function of intake but reflect the balance of dietary salt ingestion and its excretion into the urine. Regulation of sodium reabsorption from tubular filtrate in the distal nephron has a marked effect on blood pressure and although the amiloride-sensitive epithelial sodium (Na)

This article is part of the Topical Collection on *Blood Pressure Monitoring and Management*

✉ Mark Glover
mark.glover@nottingham.ac.uk

[1] Division of Respiratory Medicine, Queen's Medical Centre, University of Nottingham, Nottingham NG7 2UH, UK

[2] Division of Endocrinology, Nottingham University Hospitals NHS Trust, Nottingham, UK

[3] NIHR Nottingham Biomedical Research Centre, Nottingham, UK

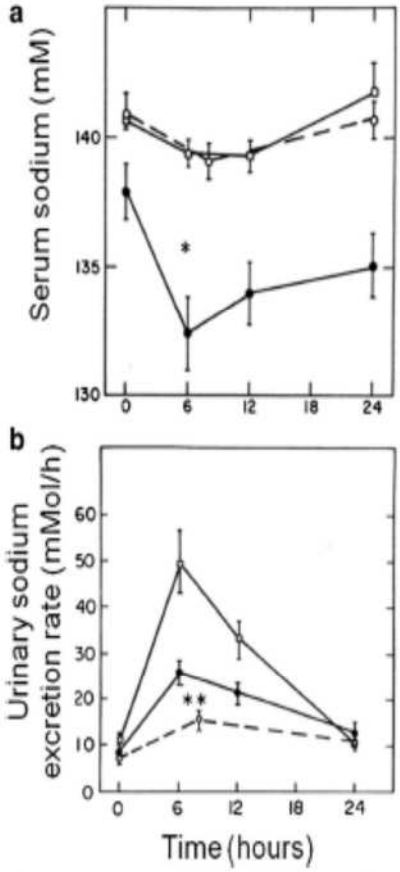


Fig. 1 TIH can occur rapidly and is accompanied by excessive saliuresis [20•]. Rechallenge of 11 patients with previous TIH (closed circles), young controls (open squares), and age-matched elderly controls (open circles) with a single oral dose of hydrochlorothiazide 50 mg plus amiloride 5 mg. Serum sodium fell within 6 h in TIH patients only (**a**) (*$P < 0.01$) and this was accompanied by excessive saliuresis relative to age matched controls (**b**) (**$P < 0.01$). Reproduced with permission from Eitan Friedman, MD, et al., Thiazide-Induced Hyponatremia: Reproducibility by Single Dose Rechallenge and an Analysis of Pathogenesis. *Annals of Internal Medicine*, Jan 01, 1989 110

Plasma glucose was slightly higher in TIH patients than in controls but at 7.6 ± 0.4 mM is insufficiently high to cause pseudohyponatremia or to be the primary cause of hyponatremia.

Spot urinary U&E in the systematic review demonstrated less than maximal urinary dilution with urinary sodium concentration at 62 mM (95% CI 45 to 80 mM) and mean urine osmolality greater than plasma at 400 mOsm/kg (95% CI 366 to 434 mOsm/kg) [17••]. The 2017 case series also showed less than minimal spot urine concentration of 31 mM and inappropriately concentrated urine with osmolality greater than plasma at 366 mOsm/Kg [18••].

The phenotype of TIH is therefore similar to the criteria for SIADH outlined by Bartter and Schwartz including (1) decreased plasma osmolality (< 275 mOsm/kg), (2) inappropriately concentrated urine (> 100 mOsm/kg), (3) euvolemia on clinical examination, and (4) elevated urine Na (> 20 mEq/L) with (5) normal thyroid and adrenal function [21]. However, as discussed below, antidiuretic hormone (ADH) is normal or low in patients with TIH.

Further Blood and Urine Phenotyping in TIH

In addition to the routine blood and spot urine tests undertaken in routine clinical care, TIH patients in the 2017 case series also underwent additional blood testing and 24 h urine collections both at the time of acute hyponatremia and after recovery to normonatremia following thiazide cessation [18••]. Hyponatremic TIH patients were also hypochloridemic, which is perhaps unsurprising since the thiazide-sensitive NCC transports both sodium and chloride. TIH cases also had a lower serum concentration of magnesium, calcium, zinc, and vitamin D relative to normonatremic controls. It is possible that the lower concentration of the non-sodium chloride electrolytes could represent a dilution of blood from water retention, and this was supported by increased fractional urate clearance, a marker of arterial blood volume expansion [22, 23]. The lower vitamin D levels in TIH cases might reflect the frailty of TIH patients who may be more limited in their diet and exposure to sunlight. Fortunately, all of these parameters improved substantially after thiazide cessation, although convalescent TIH cases showed persistence of a milder degree of hypochloridemia and hypozincemia relative to controls [18••].

TIH cases demonstrated an exaggerated free water reabsorption when taking thiazides compared to controls, which also supports a volume-expanded, diluted state. The continued production of urine which is more concentrated than plasma and which contains more than minimal sodium salt is physiologically inappropriate in the context of acute hyponatremia. Whilst 24 h urinary excretion of sodium and chloride was lower in acute TIH cases than in controls and increased from the acute state when convalescent, it is difficult to know whether such data indicates reduced salt intake in acute TIH cases given the dynamic pathophysiological state they are in. Convalescent TIH cases had slightly lower 24 h sodium and chloride excretion than controls but this was not statistically significant. There remains the possibility that TIH cases could have a slightly lower intake of dietary salt than controls but this remains unclear.

Given the similarity in phenotype between SIADH and TIH, antidiuretic hormone (ADH) was measured and was surprisingly lower in acute TIH cases than in controls or convalescent cases. The phenotype of TIH cases suggested ADH excess but in fact they had less ADH than controls, raising the question whether something other than ADH may be stimulating distal nephron water absorption.

Genetic Predisposition to TIH

Thiazide therapy does not meaningfully alter average serum sodium concentration within the treated population with essential hypertension, suggesting that there are defined subgroups of patients who are susceptible to substantial reduction in sodium on exposure to thiazides. Genetic predisposition to TIH is supported by a high degree of reproducibility on single dose thiazide rechallenge where environmental factors such as sodium intake were controlled [20•]. A priori, the molecular mechanisms underlying predisposition to TIH must result in either diminished sodium reabsorption, inappropriate water retention or a combination of the two. That there may be non-NCC effects of thiazides contributing to TIH may be argued from the absence of significant hyponatremia in Gitelman syndrome [8] or Gitelman-mimic animals carrying a loss of function mutation in the NCC regulator Ste20 Proline-Alanine-rich Kinase (SPAK) [24].

A pilot genome-wide association study (GWAS) was undertaken in a discovery cohort of 48 patients hospitalized with severe TIH and compared to patients from the 1958 British birth cohort [18••]. Given the limited number of cases available a pre-defined cut off for signals of interest showing suggestive association of $P<10^{-5}$ was used. In total, 17 SNPs within 14 regions were identified as showing association with TIH at this level. The prostaglandin E2 (PGE2) transporter (PGT, coded by the *SLCO2A1* gene), was chosen for further study because TIH has a phenotype resembling SIADH, and it was the candidate with the best known function in regulating water reabsorption in the collecting duct via the AQP2 pathway. Sequencing confirmed the presence of a non-synonymous variant encoding p.A396T (rs34550074), which was in complete linkage disequilibrium with the sentinel GWAS SNP rs4854769, and the genetic association was replicated using a second cohort of TIH cases hospitalized with severe TIH [18••].

Immunohistochemistry of human cadaveric kidneys confirmed that PGT was expressed in the collecting duct and co-localized with AQP2. Using an in vitro cell expression system, PGT containing the phospho-mimic p.A396E demonstrated loss of function. This would be expected to reduce reuptake of PGE2 from the urinary lumen and indeed increased urinary PGE2 was seen in TIH patients with the PGT p.A396T variant compared to TIH patients who were wild type, suggesting that the PGT variant may have a functional effect in patients. Differences in urinary PGE2 resolved following thiazide cessation.

Taking these findings together, a hypothesis was proposed in which the combined effect of thiazide-induced impairment of renal free water generation, together with genetically determined PGE2-mediated increased water permeability of the collecting ducts, produces a combination of natriuresis and excessive water reabsorption sufficient to lead to a substantial decline in serum sodium concentration and presentation with severe TIH (Fig. 2).

Management of TIH

The treatment of patients with TIH involves a careful balance between the desire to relieve symptoms by restoration of serum sodium and the risk of central pontine myelinolysis from overly rapid correction of serum sodium and other more general risks associated with unnecessary parenteral treatment. The balance of risks and benefits should be made by the treating physician with each patient's circumstances considered individually. We would advocate using the cautious approach adopted for chronic hyponatremia with a maximum desirable rise in serum sodium of ≤ 10 mmol/l in the first 24 h and ≤ 8 mmol/l in each following 24 h. In frail, undernourished patients, it may be prudent to be even more cautious.

Our experience of managing TIH has been that cessation of the thiazide and avoidance of excessive water intake result in recovery of serum sodium at a clinically acceptable rate of a few millimoles per liter per day in most patients. Parenteral infusion of saline under closely monitored conditions is usually reserved for TIH cases with severe or life-threatening features. In support of this approach, an observational study of thiazide -associated hyponatremia found that progressively more aggressive strategies to elevate sodium in addition to thiazide cessation (including fluid restriction, parenteral infusion of normo- or hyper-tonic saline and the use of V_2 antagonists such as tolvaptan) were found to be associated with increased probability of overly rapid correction of serum sodium [25]. It is important that patients with TIH have a serum electrolyte measurement after recovery to ensure that they are truly normonatremic. If chronic hyponatremia persists after thiazide withdrawal, then investigation for other causes of hyponatremia may be appropriate.

Conclusion

Thiazide-induced hyponatremia is an important medical condition which is seen regularly in internal medicine and which causes substantial morbidity to often elderly frail patients. At present, the risk of developing TIH is largely unpredictable at the point of thiazide initiation. TIH is also an interesting and important condition scientifically as a relatively little studied paradigm of human sodium and water dysregulation which may provide an opportunity to learn more about the molecular processes underlying these essential aspects of renal physiology.

Pressing clinical issues include:

(1) What is the optimal timing of a blood electrolyte test after starting thiazides, or should there be more than one electrolyte check?
(2) Is it possible to identify a subgroup of patients at increased risk of TIH by developing a risk score based on their age,

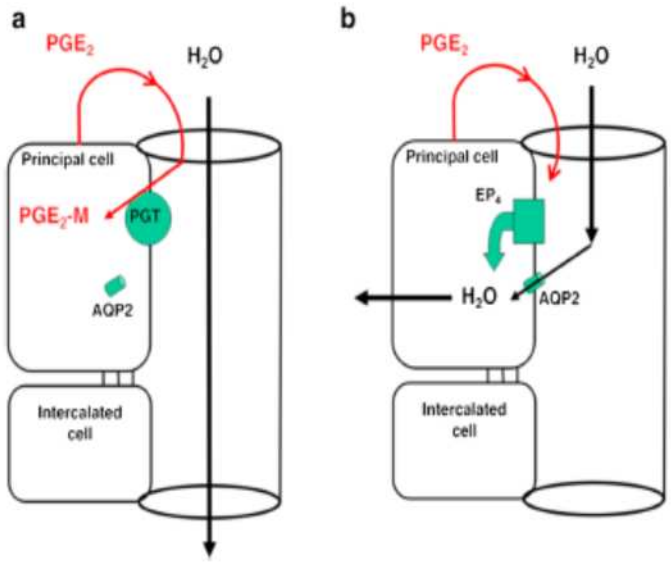


Fig. 2 Hypothesis for the role of SLCO2A1 (also known as prostaglandin transporter, PGT) in contributing to thiazide-induced hyponatremia in individuals carrying the SLCO2A1 A396T variant. **a** Under low ADH conditions, apical PGT in the renal collecting duct scavenges PGE_2 from the lumen, resulting in aquaporin-2 (AQP2) internalization and minimal osmotic water reabsorption. **b** With reduced or absent apical PGT, PGE_2 reaching the lumen is able to stimulate apical EP4 receptors, resulting in insertion of AQP2 and osmotic water reabsorption [18••]

gender, weight, baseline blood, and urine tests, and genetic profile? If so, can this be used either to select alternative antihypertensive treatment or to focus additional monitoring if such patients are exposed to thiazides?

(3) What is the optimal management of patients with TIH?

There are also many scientific questions remaining including:

(1) What causes TIH in those who do not carry the PGT p.A396T mutation?
(2) What role do the other genetic associations identified play in the pathophysiology of TIH?
(3) What effect do drugs which alter the production of PGE2 or which antagonize the EP4 receptor have on TIH pathophysiology?
(4) Is the cause of TIH consistent across ethnicities? Thiazides are used worldwide and yet most studies have been in Caucasians to date
(5) What is the physiological response to non-thiazide diuretics in those with a history of TIH?

Given the effectiveness of thiazides in lowering blood pressure and preventing cardiovascular disease and their very low cost, it seems likely that thiazide-induced hyponatremia will be an important long-term issue in medicine; there are a potentially large number of patients who may benefit from greater understanding of this condition.

Compliance with Ethics Guidelines

Conflict of Interest The authors declare no conflicts of interest relevant to this manuscript.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

References

Papers of particular interest, published recently, have been highlighted as:
• Of importance
•• Of major importance

1. N.I.C.E. Hypertension: clincial management of primary hypertension in adults. Clinical guideline 2011 [cited 2014 May 1st]; CG127:[Available from: https://www.nice.org.uk/guidance/cg127.
2. W.H.O., GLOBAL HEALTH RISKS: Mortality and burden of disease attributable to selected major risks. 2009, WHO Press: World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland p 1-70.
3. NHS. Health Survey for England -2006, CVD and risk factors for adults, obesity and risk factors for children. 2006 [cited 2018; Available from: http://digital.nhs.uk/catalogue/PUB01213.
4. Dahl L. Possible role of salt intake in the development of essential hypertension. Internation Journal of Epidemiology. 2005;34:967–72.
5. He F, MacGregor G. Effect of modest salt reduction on blood pressure: a meta-analysis of randomized trials. Implications for public health. J Hum Hypertens. 2002;16:761–70.
6. Glover M, Zuber AM, O'Shaughnessy KM. Hypertension, dietary salt intake, and the role of the thiazide-sensitive sodium chloride transporter NCCT. Cardiovasc Ther. 2011;29(1):68–76.

7. Glover M, O'Shaughnessy KM. Molecular insights from dysregulation of the thiazide-sensitive WNK/SPAK/NCC pathway in the kidney: Gordon syndrome and thiazide-induced hyponatremia. Clin Exp Pharmacol Physiol. 2013;40:876–84.
8. Simon DB, Nelson-Williams C, Johnson Bia M, Ellison D, Karet FE, Morey Molina A, et al. Gitelman's variant of Bartter's syndrome, inherited hypokalemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl cotransporter. Nat Genet. 1996;12:24–30.
9. Gordon RD. Syndrome of hypertension and hyperkalemia with normal glomerular filtration rate. Hypertension. 1986;8(2):93–102.
10. Freis E, et al. Treatment of essential hypertension with chlorothiazide (diuril): its use alone and combined with other antihypertensive agents. JAMA. 1958;166:137–40.
11. Law M, Morris J, Wald N. Use of blood pressure lowering drugs in the prevention of cardiovascular disease: meta-analysis of 147 randomised trials in the context of expectations from prospective epidemiological studies. BMJ. 2009;338:b1665.
12. The ALLHAT officers and coordinators for the ALLHAT collaborative research group. Major outcomes in high-risk hypertensive patients randomized to angiotensin converting enzyme inhibitor or calcium channel blocker vs diuretic. JAMA. 2002;288:2981–97.
13. Armstrong C. JNC8 guidelines for the management of hypertension in adults. Am Fam Physician. 2014;90(7):503–4.
14. Mancia G, et al. 2013 ESH/ESC guidelines for the management of arterial hypertension. Eur Soc Cardiol. 2013;34:2159–219.
15. Brown M. The choice of diuretic in hypertension: saving the baby from the bathwater. Heart. 2011;97:1457–551.
16. Glover M, Clayton J. Thiazide-induced hyponatremia: epidemiology and clues to pathogenesis. Cardiovasc Ther. 2012;30(5):e219–26.
17.•• Barber J, McKeever TM, McDowell SE, Clayton JA, Ferner RE, Gordon RD, et al. A systematic review and meta-analysis of thiazide-induced hyponatremia: time to reconsider electrolyte monitoring regimens after thiazide initiation? Br J Clin Pharmacol. 2015;79(4):566–77. **This is an important publication as it is the first systematic review and meta-analysis of all published TIH cases.**
18.•• Ware JS, Wain LV, Channavajjhala SK, Jackson VE, Edwards E, Lu R, et al. Phenotypic and pharmacogenetic evaluation of patients with thiazide-induced hyponatremia. J Clin Invest. 2017;127(9):3367–74. **This is an important publication as it is the first description of the pharmacogenetics of TIH and also described the phenotype of TIH in detail.**
19.• Clayton JA, Jeune IRL, Hall IP. Severe hyponatremia in medical inpatients aetiology, assessment and outcome. Q J Med. 2006;99:505–11. **This describes the aetiology of drug induced hyponatraemia in a large UK teaching hospital and found that TIH is the most frequent.**
20.• Friedman E, et al. Thiazide-induced hyponatremia. Reproducibility by single dose rechallenge and an analysis of pathogenesis. Am Coll Phys. 1989;110:24–30. **This is an important study of thiazide re-challenge in patients who have a history of TIH and showed that it is very reproducible.**
21. Bartter FC, Schwartz WB. The syndrome of inappropriate secretion of antidiuretic hormone. Am J Med. 1967;42(5):790–806.
22. Fenske W, Störk S, Koschker AC, Blechschmidt A, Lorenz D, Wortmann S, et al. Value of fractional uric acid excretion in differential diagnosis of hyponatremic patients on diuretics. J Clin Endocrinol Metab. 2008;93(8):2991–7.
23. Sonnenblick M, Rosin AJ. Significance of the measurement of uric acid fractional clearance in diuretic induced hyponatremia. Postgrad Med J. 1986;62:449–52.
24. Rafiqi FH, et al. Role of the WNK-activated SPAK kinase in regulating blood pressure. EMBO Mol Med. 2009;2:63–75.
25. Burst V, Grundmann F, Kubacki T, Greenberg A, Becker I, Rudolf D, et al. Thiazide-associated hyponatremia, report of the hyponatremia registry: an observational multicenter international study. Am J Nephrol. 2017;45(5):420–30.

9.3 Ethical approval

9.3.1 Thiazide-Induced Hyponatraemia Ethical Protocol

The University of Nottingham

The Pharmacogenetics of Thiazide Induced Hyponatraemia

Final Version 1.8
03 October 2017

Short title:	The Genetics of Salt Imbalance on Thiazide Diuretic Medication
Acronym:	TPTIH
NRES reference:	11/EM/0233
Trial Sponsor:	University of Nottingham
Funding Source:	1) Medical Research Council (Awarded) 2) Nottingham University Hospitals Charity (Awarded)

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

STUDY PERSONNEL AND CONTACT DETAILS

Sponsor:
Contact name

University of Nottingham
Mr Paul Cartledge
Head of Research Grants and Contracts
Research Innovation Services
King's Meadow Campus
Lenton Lane
Nottingham
NG7 2NR

Chief investigator:

Professor Ian P. Hall
Division of Respiratory Medicine,
D Floor, South Block,
Queen's Medical Centre,
Nottingham
NG7 2UH
Phone: +44 (0)115 82 31063
Fax: +44 (0)115 82 31059
Email: ian.hall@nottingham.ac.uk

Co-investigator:

Dr Mark Glover
Division of Respiratory Medicine,
D Floor, South Block,
Queen's Medical Centre,
Nottingham
NG7 2UH
Phone: +44 (0)115 82 31063
Fax: +44 (0)115 82 31059
Email: mark.glover@nottingham.ac.uk

Trial / Study Coordinating Centre:

Division of Respiratory Medicine,
University of Nottingham,
D Floor, South Block,
Queen's Medical Centre,
Nottingham,
NG7 2UH

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

STUDY SYNOPSIS

Title	The Pharmacogenetics of Thiazide Induced Hyponatraemia
Acronym	TPTIH
Chief Investigator	Professor Ian P Hall
Study centre(s)	Nottingham University Hospitals NHS Trust Cambridge University Hospitals NHS Foundation Trust Primary care practises to be determined *Insert additional?*
Objectives	1) To determine whether there is a genetic basis for thiazide-induced hyponatraemia. 2) If so, which genes are responsible?
Number of participants / tissue samples	Up to 600
Diagnosis and main criteria for inclusion	Inclusion criteria: 1) Age 18-100 years 2) Have the capacity to give informed consent 3) Be taking a thiazide or thiazide-related diuretic unless in the non-thiazide control group, or **an SSRI or anti-convulsant in the non-thiazide drug induced hyponataremia control group** 4) Plasma sodium concentration of equal to or less than 130 mM for cases and between 135 mM and 145 mM for controls
Duration of study	Overall: *Planned start date 01 September 2011* Per participant: 5 minutes for consent 2x5 minutes for 2 venepunctures 5 minutes each for two small spot urine samples 2x 24 hour urine collections
Description of interventions	Two venepunctures or exceptionally, a saliva collection Two small urine samples Two 24 hour urine collections
Statistical methods	Association testing only

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

Table of contents

STUDY PERSONNEL AND CONTACT DETAILS 2
STUDY SYNOPSIS 3
ABBREVIATIONS 5
STUDY BACKGROUND INFORMATION AND RATIONALE 5
Summary 5
STUDY OBJECTIVES AND PURPOSE 14
PURPOSE 14
PRIMARY OBJECTIVE 14
SECONDARY OBJECTIVES 14
STUDY DESIGN 14
STUDY CONFIGURATION 14
Primary endpoint 15
Secondary endpoint 15
TRIAL MANAGEMENT 15
DURATION OF THE TRIAL / STUDY AND PARTICIPANT INVOLVEMENT 15
End of the Trial 15
SELECTION AND WITHDRAWAL OF STUDY PARTICIPANTS 16
Recruitment 16
Inclusion criteria 17
Exclusion criteria 17
Informed consent 17
TRIAL / STUDY TREATMENT AND REGIMEN 17
Sample size and justification 18
ADVERSE EVENTS 18
TRANSPORT AND STORAGE OF THE TISSUES 18
LABORATORY ANALYSES 19
STATISTICS 20
Statistical analysis 20
Primary Outcome Measure 20
ETHICAL AND REGULATORY ASPECTS 20
Ethical Issues 20
Informed Consent and Participant Information 20
Withdrawal 21
RECORDS 21
Source data / documents 21
Sample Labelling 21
Source documents 22
Direct access to source data / documents 22
Data Protection 22
QUALITY ASSURANCE & AUDIT 22
INSURANCE AND INDEMNITY 22
STUDY CONDUCT 22
STUDY DATA 23
RECORD RETENTION AND ARCHIVING 23
DISCONTINUATION OF THE STUDY BY THE SPONSOR 23
STATEMENT OF CONFIDENTIALITY 23
PUBLICATION POLICY 23
STUDY FINANCES 24

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

Funding source 24
Participant stipends and payments..... 24
REFERENCES..... 25

ABBREVIATIONS

AATYK Apoptosis-Associated TYrosine Kinase
AC Adenylate Cyclase
ADH AntiDiuretic Hormone
AP PKA anchoring proteins
AQP Aquaporin
AT1R Angiotensin 1 Receptor
ATP Adenosine triphosphate
cAMP cyclic Adenosine MonoPhosphate
CLCNKB Basolateral membrance chloride transporter
COX Cyclo-OXygenase
ER Endoplasmic Reticulum
Gαs G-protein alpha subunit
GWAS Genome Wide Association Study
KCNJ10 ATP-sensitive inward rectifier potassium channel 10
MR Mineralocorticoid Receptor
NCC Thiazide-sensitive sodium chloride transporter
NKCC2 Loop diuretic sensitive sodium transporter
NHE3 Proximal tubule sodium transporter
OAT Organic Anion Transporter,
PCT Proximal Convoluted Tubule
PG ProstaGlandin
PHAII PseudoHypoAldosteronism type 2
PKA Protein kinase A
PP4 Protein Phosphatase 4
ROMK Renal Outer Medullary potassium transporter
SPAK STE-20/SPS1 proline/alanine-rich kinase
TAL Thick Ascending Loop of Henle
TIH Thiazide Induced Hyponatramia
TGN Trans Golgi Network
V2R Vasopressin 2 receptor
WNK With No Lysine (K) kinase

STUDY BACKGROUND INFORMATION AND RATIONALE

Summary

Thiazide diuretics are one of the most widely used and cost-effective class of anti-hypertensive agents worldwide. Thiazides however have a significant side effect profile and are frequently insufficient to normalise blood pressure alone. Thiazide induced hyponatraemia (TIH) is a major adverse effect, affecting up to one in seven patients receiving these drugs. TIH is more common in females, the elderly and those of low body weight and may cause symptoms such as confusion, falls and seizures. It is a common cause of hospital admission in the elderly.

Although TIH occurs at least as frequently as hypokalaemia, much less is understood about its mechanism. Thiazides lower blood pressure by reducing the reabsorption of

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

sodium from the distal nephron by inhibition of the Na-Cl cotransporter, NCC. The molecular mechanism by which this occurs together with the less appreciated role of thiazides in regulating water reabsorbtion from the collecting ducts is discussed and the relevance to TIH evaluated.
TIH is highly reproducible by thiazide rechallenge suggesting there may be a genetic predisposition. Both targeted resequencing of candidate genes and genome wide association techniques offer promising strategies by which such genetic contributions may be investigated.

The rewards for uncovering the molecular mechanisms underlying TIH and the regulation of distal nephron sodium and water absorption are significant; not only could it inform the design of better tolerated, more efficacious thiazide-like antihypertensive agents but it may also facilitate the pharmacogenomic profiling of hypertensive patients to avoid thiazides in those likely to suffer adverse effects.

Background
Thiazide diuretics are one of the oldest, most widely prescribed and cost-effective class of antihypertensive medication worldwide [1;2] with all cause mortality benefits equal to angiotensin-converting enzyme inhibitors and calcium channel antagonists [3]. Thiazides however are no panacea; they have a significant side effect profile. Some adverse effects are directly related to inhibition of the thiazide-sensitive Na-Cl cotransporter (NCC) in the distal convoluted tubule such as hypokalemia, hypercalcaemia and possibly hypomagnesemia and hyopnatraemia, but most appear to be idiosyncratic off-target effects including gout, photosensitization, impotence, and impaired glucose tolerance [4]. Hence, a better understanding of the molecular mechanisms that are responsible for the adverse effects of thiazide diuretics should aid the design of novel antihypertensive diuretics with fewer side effects and could even lead, through pharmacogenomic profiling, to the personalised prescribing of antihypertensive medications.

Epidemiology
Thiazide-induced hyponatraemia (TIH) is less well characterised than hypokalaemia yet occurs at least as frequently, affecting up to 14% of patients prescribed thiazides in primary care [5]. Hyponatraemia is important in hospitalised patients too; it is the most frequent electrolyte abnormality observed amongst medical in-patients and affects up to a third of elderly patients taking thiazides on admission [6;7]. For patients hospitalised with severe hyponatraemia (sodium <125 mM) the role of thiazide diuretics is even more stark: thiazides are responsible for over a quarter of such presentations and are by far the most common cause of drug-induced severe hyponatraemia [8].

Large scale prospective trials of thiazides have not demonstrated a uniform reduction in serum sodium concentration [9;10] and yet some individuals are clearly affected, implying that there may be subgroups of the treated population who are susceptible [5;11]. Risk factors predisposing to TIH include:

- increasing age [5;11-15] with those over 70 years carrying a threefold higher relative risk, possibly due to age related reduction in free water clearance [16]
- low body weight [11]
- hypokalaemia [11]
- female gender [12;13;17], although this may be confounded by the over representation of female patients in thiazide-treated cohorts [11].

Clinical presentation
The onset of thiazide-induced hyponatraemia is typically within 2 weeks [18] however in some individuals plasma sodium can fall within hours of thiazide ingestion and severe hyponatraemia can develop in less than 2 days [12;13]. TIH may less commonly develop after months or years [11] when additional factors such as polypharmacy, co-morbidities

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

and decline in renal function may contribute. Although hyponatraemia is a continuous variable it may be usefully classified into mild (Na^+ 134-131 mmol/l), moderate (Na^+ 125-130 mmol/l) or severe (Na^+<125mmol/l).

Symptoms depend on the speed of onset and degree of hyponatraemia. Patients with a sodium concentration above 125 mmol/l are often asymptomatic although may experience gait and attention deficits with increased risk of falls [19] and fractures [20]. Once sodium drops below 120mmol/l most patients have significant symptoms [21] including nausea and vomiting, muscular weakness, headache, confusion and ataxia. Severe and rapidly evolving hyponatraemia induces cerebral oedema and can lead to seizures, coma, brain damage, respiratory arrest, brain stem herniation and death [22;23].

The majority of patients with TIH are clinically euvolaemic with low or normal plasma urea, creatinine, urate and potassium [24]. Urinary osmolality is high relative to plasma and often exceeds it [12;25;26]. Urinary sodium concentration is usually greater than 20 mmol/l [25;26].

Differential diagnosis of TIH

The aetiology of hyponatraemia is often multifactorial [8] and other causes or contributing factors need to be considered (Table 1). A careful assessment of volume status is important both for diagnosis and management.

Impaired capacity of renal water excretion or sodium reabsorption		
Hypovolaemic	**Euvolaemic**	**Hypervolaemic**
Renal sodium loss	Thiazide diuretics	Congestive heart failure
Diuretics	Hypothyroidism	Cirrhosis
Hyperglycaemia	Glucocorticoid deficiency	Nephrotic syndrome
Adrenal insufficiency	SIADH	Renal failure
Salt-wasting nephropathies	Malignancy	Pregnancy
Cerebral salt wasting		
Extrarenal sodium loss		
Diarrhoea		
Vomiting		
Haemorrhage		
Excessive sweating		
Third spacing of fluids		
Burns		
Pancreatitis		
Bowel obstruction		
Excessive water intake		
Primary polydipsia		
Sodium-free irrigant solutions		
Inappropriate intravenous fluid replacement (e.g. 5% Dextrose solution)		

Table 1: Causes of hyponatraemia. Syndrome of Inappropriate Anti-Diuretic Hormone (SIADH).

Management of thiazide-induced hyponatraemia

There is little consensus on electrolyte monitoring in patients prescribed a thiazide or on the management of TIH. As the onset of TIH is usually within 1-2 weeks [18] it is prudent to advise a check sodium concentration after 2 weeks of treatment and annually thereafter.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

If hyponatraemia occurs management will depend on the sodium concentration, the presence of symptoms and indication for the thiazide.

Mild and moderate hyponatraemia (sodium >125 mmol/l) – Thiazide cessation and replacement with an alternative antihypertensive agent is desirable. TIH usually corrects within 1 to 5 days of drug cessation but can take up to 2 weeks [27]. However in asymptomatic patients this needs to be balanced against the potential risk of poorly treated hypertension if other agents fail to control blood pressure adequately. In moderate hyponatraemia (Na<130) fluid restriction is sometimes advocated [28].

Severe hyponatraemia (sodium <125 mmol/l) - These patients are likely to be symptomatic and are often managed in hospital. A delicate balance must be struck between the of risks of cerebral oedema and the risks of osmotic demyelination syndrome related to the rate of correction of the hyponatraemia [29]. In the absence of severe manifestations such as seizures or coma conservative treatment (thiazide cessation) is advised. The majority of patients will be euvolaemic but if hypovolaemic, normal saline should be infused. In the presence of severe manifestations (seizures or coma) management in a high dependency or critical care setting is required. Hypertonic (3%) saline may be given cautiously with frequent electrolyte monitoring (every 2-3 hours); formulae can be used to guide the infusion rate [30]. Relatively small increases in serum sodium concentration (3 to 7 mmol/l) substantially reduce cerebral oedema [31] and often terminate seizures [32;33]. An initial correction rate of 1-2 mmol/l/hour is advised for the first few hours but thereafter, to reduce the risk of osmotic demyelination syndrome, the rate of correction should be slowed aiming for a daily correction rate of no more than 12 mmol/l [22].

Pathophysiology

Despite careful clinical and biochemical characterisation and the identification of risk factors predisposing to TIH, little is understood of the molecular mechanisms by which hyponatraemia occurs. Unlike hypokalaemia, hyponatraemia is dose-independent [5;8] suggesting that this may be an idiosyncratic effect. Although some individuals develop marked hyponatraemia, the average plasma sodium concentration within the thiazide treated population is unchanged [5] implying that there may be defined subgroups of patients who are susceptible.

Moreover, recurrence of hyponatraemia is highly reproducible in patients with previous TIH by single dose thiazide rechallenge [34]. This suggests that there may be genetic predisposition to the development of TIH, given the controlled dietary conditions of the study population.

A priori, the molecular mechanisms underlying a possible genetic predisposition to TIH must either result in diminished sodium reabsorption, inappropriate water retention or a combination of the two and suggests that defective gene products may reside in the distal nephron.

Mechanisms of sodium reabsorption

The kidneys filter some 170 litres of plasma daily containing around 23 moles of sodium, of which over 99.5% is reabsorbed by a series of sodium transporters along the nephron (Figure 1). The greatest fraction of filtered sodium (60-70%) is reabsorbed in the proximal convoluted tubule (PCT) by the Na^+/H^+ exchanger NHE3 [35].

A further 20-30% is reabsorbed in the Thick Ascending limb of the Loop of Henle (TAL) by the Na-K-2Cl transporter NKCC2, the target of Loop diuretics such as furosemide [36]. Potassium is recycled across the apical membrane, in a rate limiting manner, by the renal outer medulla potassium channel, ROMK, whereas Cl^- exits the cell via the basolateral membrane through chloride channels and transporters including CLCNKB. Mutations within any of these genes (NKCC2, ROMK, and CLCNKB) leads to Bartter syndrome: type

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

I, II, and III respectively [37-40]. Bartter syndrome is a salt wasting condition, mimicking treatment with Loop diuretics and is characterized by hypokalemia, metabolic alkalosis, hypercalciuria and a tendency to hypotension.

Of the remaining 10% of sodium reaching the distal nephron, 5-7% is reabsorbed by the thiazide-sensitive Na-Cl cotransporter, NCC in the distal convoluted tubule (DCT). The DCT is the site of two other monogenic diseases. First, loss of-function mutations within NCC lead to Gitelman syndrome [41], which resembles therapy with thiazide diuretics. Second, Gordon syndrome [42] (pseudohypoaldosteronism type 2 or PHAII), the clinical mirror of Gitelman syndrome results from increased NCC membrane expression; it is characterized by hypertension and hyperkalemic acidosis and caused by mutations within one of two members of a novel family of regulatory kinases called the "With No lysine (K)" kinases (WNK1 and WNK4) [43;44].

A final 2-3% of filtered sodium is reabsorbed in the connecting tubule (CNT) and the collecting duct (CD) by the amiloride-sensitive epithelial sodium channel, ENaC. Gain of function mutations in ENaC cause Liddle syndrome, a condition of hypokalaemic alkalosis and low renin hypertension [45]. Conversely loss of function ENaC mutation leads to an autosomal recessive syndrome of salt wasting termed pseudohypoaldosteronism type 1 [46].

ENaC regulation by aldosterone is reflected by syndromes of hyper- and hypo-tension caused by gain- and loss-of function mutations in the mineralocorticoid receptor: the syndrome of activating mineralocorticoid receptor mutation [47] and a mild form of autosomal dominant pseudohypoaldosteronism type 1 respectively [48].

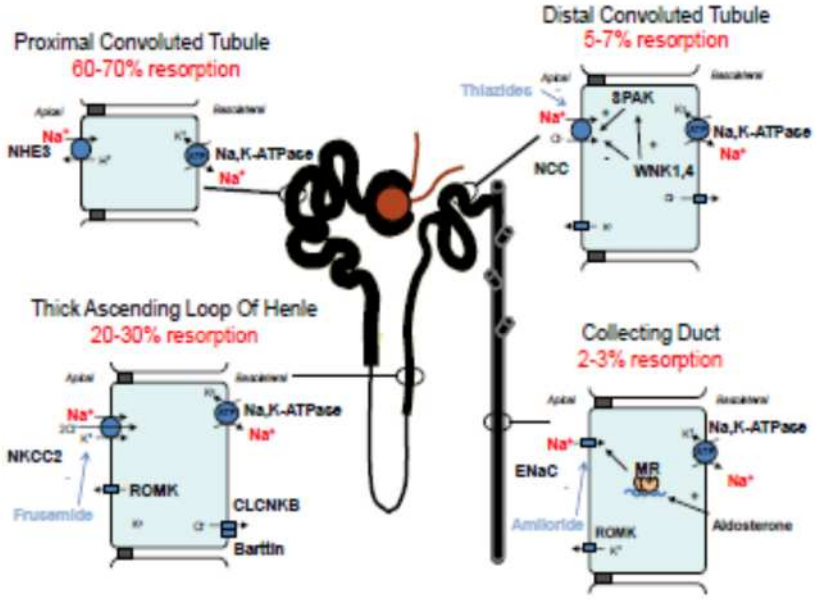


Figure 1
Schematic representation of the nephron showing principal sites of sodium reabsorption. In the proximal convoluted tubule via NHE3, Na^+/H^+ exchanger isoform 3; in the thick ascending Loop of Henle via the Frusemide-sensitive NKCC2, $Na^+/K^+/2Cl^-$ cotransporter; in the distal convoluted tubule by the thiazide-sensitive NCC, Na^+/Cl^- cotransporter and in the collecting duct via the amiloride-sensitive epithelial sodium channel (ENaC). ROMK (renal outer medulla potassium channel), CLCNKB (chloride channel kidney B), SPAK

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

(STE-20/SPS1 proline/alanine-rich kinase), WNKs 1/4 (with no lysine kinases 1/4) and MR (mineralocorticoid receptor) are also shown.

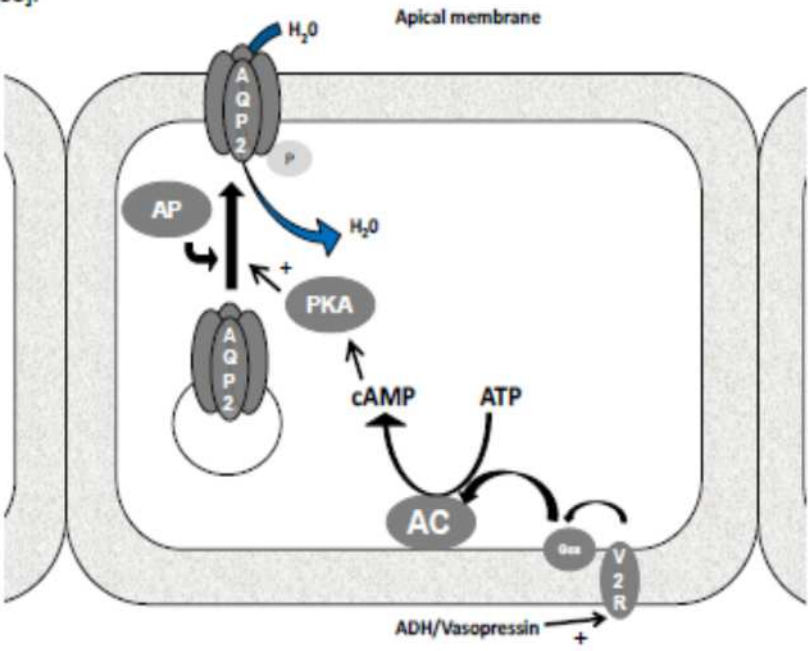
Mechanisms of water reabsorption

Osmotically driven water reabsorption through renal tubules and the vasa recta is orchestrated by a family of water-permeable transporters, the aquaporins (AQPs). First characterised by Agre in 1992 [49] there are now 11 identified isoforms of this transporter, each with subtly different properties and expression patterns. Some are only permeable to water (AQPs 0,1,2,4,5 and 6) whilst others are also permeable to ammonia (AQPs 3,7,8,9, and possibly 10) or urea (AQP 7, 9 and possibly 3) [50].

Around 90% of glomerular filtrate is reabsorbed in the proximal convoluted tubule and descending Loop of Henle via AQP1 which is expressed in apical and basolateral membranes of the proximal nephron and vasa recta [51]. Whilst the proximal tubule has constitutively high water permeability, the ascending LoH is relatively impermeable to water which leaves the distal nephron, in particular the principal cells of the collecting duct, to regulate solute-free water excretion.

Water reabsorption in the collecting duct is under the control of anti-diuretic hormone (ADH/vasopressin). AQP2 is the principal ADH-regulated aquaporin and is expressed in the apical membrane, subapical vesicles and basolateral membrane of principal cells [52]. ADH binds to and activates V2 receptors on the basolateral membrance of principal cells, causing a rise in intracellular cAMP by activation of the GTP-binding subunit Gαs (Figure 2). This activates Protein Kinase A (PKA) which is then recruited to sub-apical membrane vesicles containing AQP2 by PKA-anchoring proteins [53]. PKA then phosphorylates AQP2 at Serine 256 causing vesicular fusion and thus AQP2 expression in the apical membrane. ADH also affects long term AQP2 regulation by changes in protein expression [54].

The importance of the ADH-AQP2 system to human water regulation is underlined by nephrogenic diabetes insipidus resulting from mutation in either the V2 receptor (X-linked) or AQP2 (in which separate mutations result in either autosomal dominant or recessive forms) [55].



This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

Figure 2
Schematic representation of a principal cell in the collecting duct showing water reabsorption by the vasopressin-aquaporin system. ADH (anti-diuretic hormone also known as vasopressin), V2R (vasopressin 2 receptor), Gαs (G-protein alpha subunit), AC (adenylate cyclase), ATP (adenosine triphosphate), cAMP (cyclic adenosine monophosphate), PKA (protein kinase A), AP (PKA anchoring proteins) and AQP2/AQP2P (aquaporin subunit 2 in non-phosphorylated and phosphorylated forms respectively) are shown.

Possible Molecular Mechanisms underlying TIH
Disordered NCC regulation?

Much has been learnt regarding the regulation of the thiazide-sensitive Na-Cl cotransporter, NCC, from the study of monogenic syndromes of loss of function (Gitelman) or gain of function (Gordon) mutations and these reflect the molecular mechanisms of NCC regulation detailed *in vitro* (Figure 3).

Type 1 Gitelman mutations encode transporters which fail to be trafficked to the apical membrane and are retarded in the endoplasmic reticulum (ER) or Golgi apparatus [56], resulting in a reduced maximum transporting capacity (Bmax). Conversely, *Type 2* NCC mutations encode transporters which are trafficked to the membrane but which display reduced transporter activity (Vmax) [56].

Type 1 regulation of NCC, a trafficking effect is mediated *in vitro* by the WNK kinases mutated in Gordon syndrome (WNKs 1&4) and also WNK3. At least for wild type WNK4 this seems to involve AP3 mediated lysosomal NCC trafficking [57].

Type 2 NCC regulation (activity regulation) is determined by the phosphorylation of N-terminal Ser/The residues [58;59]. Human NCC T60 (homologous to mouse T58) appears most important, reflected by T60M mutation causing Gitelman syndrome in some pedigrees [60]. STE20-related Proline Alanine rich Kinase (SPAK), phylogenetically related to the WNK kinases, has recently been recognised as the effector of *type 2* regulation and a potential antihypertensive drug target [56]. Intronic, gain of function SPAK mutations, are associated with human hypertension [61], whereas kinase-inactivating, loss of function SPAK mutation causes a salt wasting hypotensive phenotype akin to Gitelman syndrome in knock-in mice [62].

It is possible that NCC or WNK mutation could predispose to TIH for example by producing thiazide hypersensitivity and augmenting thiazide-induced distal nephron sodium loss. Unpublished preliminary work within our group used Sanger sequencing using genomic DNA from a small number of affected patients (Na^+<125mM) and matched controls. Specifically sequencing of all 26 NCC exons and WNK4 exon 7 (encoding the acidic motif in which most PHA2 mutations are clustered) was undertaken [63]. Although rare as well as common polymorphisms within NCC and WNK4 were identified this failed to reach statistical significance. The most promising SNP (T465C) was located within NCC exon 11 with an odds ratio of 2.1, p=0.03.

However, NCC regulation has emerged as significantly more complex than first appreciated and is orchestrated by a scaffold of interacting proteins [4] including several new NCC regulators such as apoptosis-associated tyrosine kinase (AATYK) [64], protein phosphatase 4 (PP4) [65], ATP-sensitive inward rectifier potassium channel 10 (KCNJ10) [66], γ-adducin [67] and the angiotensin 1 receptor (AT1R) [68] whose genes would now also be candidates for investigation.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

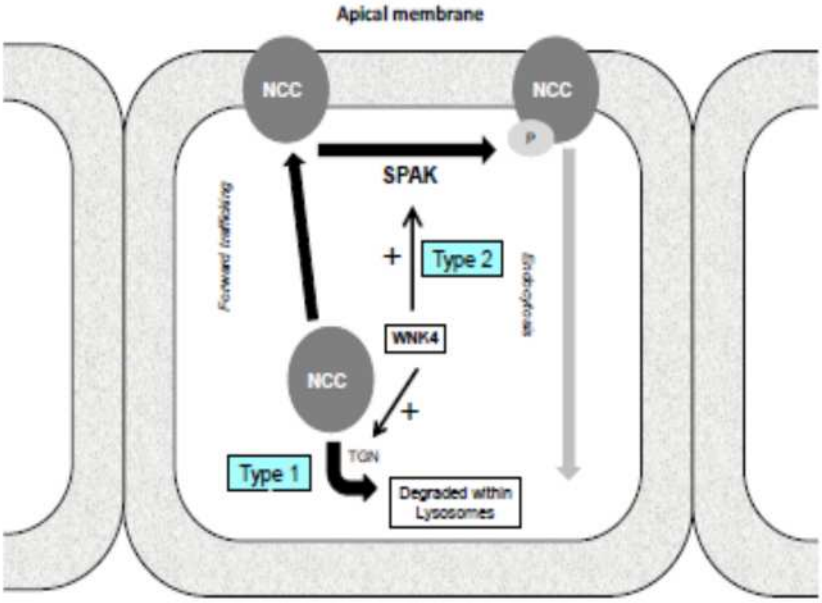


Figure 3
Schematic representation of the two principal mechanisms of regulation of the thiazide-sensitive Na^+/Cl^- cotransporter, NCC. *Type 1* regulation affects forward trafficking of NCC to the apical membrane. At least for wild type WNK4 this appears to be by diversion of NCC through the trans-golgi network (TGN) for degradation in lysosomes. *Type 2* regulation of NCC determines intrinsic transporter kinetics by phosphorylation of key N-terminal serine/threonine residues such as T58. This phosphorylation is mediated by STE-20/SPS1 proline/alanine-rich kinase (SPAK) which in turn is activated by WNK4.

Disordered aquaporin regulation?
It is possible that TIH patients may tend towards excessive water consumption or reabsorption from the distal nephron; although biochemical parameters closely resemble SIADH there are no reproducible differences in ADH secretion [34]. Patients who develop TIH display reduced free water clearance, impaired maximum urine dilution [16] and acute weight gain on single dose thiazide rechallenge [34].

Microperfusion studies suggest that thiazides induce acute upregulation of AQP2 when applied to the apical but not basolateral membrane of collecting duct cells, increasing their water permeability [69;70]. Thiazide-induced water permeability is inhibited by the functional ADH antagonist PGE2 or inhibitors of PKA (presumably by countering the ADH mediated rise in intracellular cAMP) and augmented by indomethacin [69;70]. This correlates well with clinical observations of an indomethacin-induced reduction in free water clearance of healthy controls as well as the potentiation of thiazide-induced serum hypo-osmolarity by indomethacin polypharmacy [16].

The possibility that non-NCC effects of thiazides account for TIH may be argued from the absence of clinically significant hyponatraemia in Gitelman syndrome [71]. Indeed the literature on severe hyponatraemia in Gitelman syndrome consists of a solitary patient after prolonged polydypsia [72].

Rationale for our research into the genetics of TIH
That particular individuals within the thiazide-treated population develop hyponatraemia which is highly reproducible on single-dose thiazide rechallenge [34] makes genetic

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

analysis of such individuals a promising strategy to uncover the molecular mechanisms underlying this disorder.

There are two principal strategies which we shall use to investigate the genetic basis of TIH; targeted resequencing of candidate genes and Genome Wide Association Study.
Targeted resequencing using high throughput next generation techniques with deep coverage permits investigation of hitherto prohibitive numbers of patients and candidate genes given the rapidly reducing cost of this technology. A limited number of candidate genes have been identified; these include NCC and SPAK, which mediate thiazide-sensitive sodium reabsorption from the distal convoluted tubule, AQP2 and the V2 (vasopressin/ADH) receptor, which regulate water absorption in the collecting duct and the organic anion transporter, OAT1. The latter is included as thiazides, largely bound to plasma proteins, are actively secreted into the proximal tubule by OAT1, a protein with well described functional polymorphism [75].

This strategy has the potential to identify rare variants in these genes. Rare genetic variants are predicted to be enriched for functional alleles and to exhibit stronger effect sizes than common variants as a consequence of the purifying selection pressure they are subject to. That rare polymorphisms contribute to human phenotypes is well established as illustrated by the effect that such polymorphisms in renal sodium transporters, including NCC, have on human blood pressure [73]. Next generation resequencing, by identifying rare polymorphisms, is already starting to show results in the field of lipid regulation [74].

There are however several disadvantages to a candidate gene approach, albeit with the aid of higher throughput next generation sequencing techniques. Firstly, for all the recent advances in understanding NCC and aquaporin regulation there is much that remains unknown. Thus even an expanded and potentially lengthy list of candidate genes containing all known regulators of NCC and aquaporins may not contain the causative variant. Moreover, the critical polymorphism may be in non-NCC/AQP mediated sodium and water pathways such as reduced downstream sodium absorptive capacity in the collecting ducts.

Secondly a list of candidate genes should also include those which mediate thiazide pharmacokinetic pathways, many of which are poorly understood. Thiazide pharmacokinetics vary significantly between agent; bioavailability varies between 60% (hydrochlorothiazide) and 90% (bendroflumethiazide), most are at least 95% bound to plasma proteins and, being sulphonamide derivatives, are excreted into the urine via the organic acid transporter, SLC22A6 (OAT1) in the proximal convoluted tubule [75]. The degree to which thiazides are excreted unchanged in the urine also varies widely from 100% for hydrochlorothiazide to as little as 30 % for bendroflumethiazide. The remaining 70% of bendroflumethiazide undergoes clearance by a poorly characterised metabolic pathway [76].

Thirdly, many of the candidate genes are large eg WNK1 and polymorphisms producing phenotypic variance such as blood pressure are intronic (e.g. as seen with SPAK[61] and WNK1[77]) such that sequencing of all candidate genes, both introns and exons, may be inefficient.

Therefore we have considered that whole genome techniques such as whole exome sequencing or Genome Wide Association Study (GWAS) may also be of considerable importance and address some of the weaknesses of targeted resequencing. Whilst whole exome sequencing would address coding regions only GWAS could potentially uncover intronic variants. GWAS has enjoyed considerable success in identifying pharmacogenomic polymorphisms responsible for a variety of adverse drug phenomenon; examples include simvastatin induced myopathy due to polymorphism of the hepatic anion-transporter SLO1B1(OATP1B1) [78] and HLA haplotype in association with

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

lumaricoxib induced hepatitis [79], amoxicillin-clavulanate induced cholestasis [80;81], carbamazepine induced Stevens-Johnson syndrome [82], abacavir hypersensitivity [83] and ximelagatran induced hepatitis [84].

Whichever technique is successful (and both may be in differing ways) uncovering the mechanisms underlying thiazide-induced hyponatraemia remains a pressing issue. Not only would an understanding of the molecular details of the mechanisms involved aid the design of new antihypertensive thiazide-like drugs but it would bring Medicine one step closer to an era of individualised prescribing based on pharmacogenomic profiling [85].

STUDY OBJECTIVES AND PURPOSE

PURPOSE

PRIMARY OBJECTIVE

To establish genetic associations between patients with thiazide-induced hyponatraemia and patients taking a thiazide who did not develop hyponatraemia.

SECONDARY OBJECTIVES

To detail the genetic loci and polymorphisms associated with thiazide-induced hyponatraemia.

To better characterise the plasma and urinary electrolyte profile of patients with TIH compared to controls.

To characterise the urinary sodium transporter profile in patients with TIH and controls.

STUDY DESIGN

STUDY CONFIGURATION

The study shall be managed from the University of Nottingham but shall recruit from Nottingham University Hospitals NHS Trust, *Cambridge University Hospitals NHS Foundation Trust, primary care practises in the Midlands and in the longer term potentially the GPRD (General Practise Research Database) and other centres if necessary. If we are successful in obtaining funding from the Academy of Medical Sciences then we will also recruit centres via the NIHR UKCRN (National Institute for Health Research United Kingdom Clinical Research Network).*

We propose to conduct a case controlled, targeted resequencing study and a Genome Wide Association Study (GWAS) to characterise genetic polymorphisms associated with thiazide-induced hyponatraemia. These genetic approaches have been successful in understanding how genetic variation contributes to blood pressure and cholesterol regulation at a molecular level. It is hoped a similar approach for thiazides will improve the design of new blood pressure lowering drugs and allow predication of who is likely to get low sodium by genetic testing.

We shall also characterise the phenotype of thiazide-induced hyponatraemia by measurement of 24 hour urinary electrolytes, endogenous sodium and lithium clearance and abundance of sodium transporters in the urine in patients and controls.

This research proposal has been developed by Professor Ian Hall (Chair of Therapeutics and Professor of Clinical Pharmacology) and Dr Mark Glover (Associate Professor and Honorary Consultant Physician).

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

The research protocol is subject to peer review from the Academy of Medical Sciences, Nottingham University Hospitals Charity and the University of Nottingham Early Careers Knowledge Transfer award committee who may each fund an aspect of the genetic research from a common recruitment process. We have also consulted patients regarding the design of the study and consent procedures. Patients have been consulted both in hospital who suffer from thiazide-induced hyponatraemia and in hypertension clinic who are taking a thiazide diuretic but who do not currently have hyponatraemia.

Recruitment

Timescale

Recruitment will begin in September 2011 or as soon after this date that approval is granted and will continue for 11 years.

Primary endpoint

Collection of blood samples from which DNA shall be extracted.

Secondary endpoint

Genetic analysis to determine associations with thiazide-induced hyponatraemia by means of targeted resequencing and Genome Wide Association Study.

Characterisation of the plasma and urinary electrolyte profile of patients with TIH compared to controls.

Characterisation of the urinary sodium transporter profile in patients with TIH and controls

TRIAL MANAGEMENT

This research proposal has been developed by Professor Ian Hall (Chair of Therapeutics and Professor of Clinical Pharmacology) Dr Jenny Clayton (Consultant Endocrinologist) and Dr Mark Glover (Associate Professor and Honorary Consultant Physician).

All aspects of recruitment, storage and analysis of samples and the resulting genetic association techniques shall be overseen by Professor Hall and Dr Mark Glover with results submitted for publication in peer reviewed journals as well as presented at key national and international scientific meetings.

The Chief Investigator has overall responsibility for the study and shall oversee all study management.

DURATION OF THE TRIAL / STUDY AND PARTICIPANT INVOLVEMENT

In hospital the blood sample may be taken immediately after the consent i.e. on the same day unless the patient requests otherwise. Urine samples will be taken contemporaneously on the day of the blood sample and again when convalescent either shortly before discharge from hospital or shortly after discharge from hospital.

In primary care it may take several days or weeks to arrange an appointment to discuss consent and arrange for blood and urine samples to be taken.

End of the Trial

Recruitment of the last participant in September 2017. Genetic and other analysis will continue with a timetable determined by the technology and research findings.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

SELECTION AND WITHDRAWAL OF STUDY PARTICIPANTS

Recruitment

Participants will be recruited from general medicine emergency admissions, medical outpatient clinics and general practise surgeries. The initial approach will be from a member of the patient's usual care team (which may include the investigator).

The investigator or their nominee, e.g. from the research team or a member of the participant's usual care team, will inform the participant or their nominated representative (other individual or other body with appropriate jurisdiction), of all aspects pertaining to participation in the study.

In hospital, patients and matched controls shall be recruited by a medical registrar, consultant or research nurse. Patients will be identified from the medical take or outpatient clinics by liaison with the responsible physicians and identification from biochemistry computer databases in hospital. The initial approach shall be by one of the patient's usual care team and during the process of ensuring eligibility for inclusion in the study relevant information about the patient's medical problems and other medication shall be recorded from the patient and from the medical records. Matched controls (i.e. patients taking a thiazide diuretic but with normal sodium) shall be recruited from the same sources.

Following identification informed consent will be sought and if the patient is in agreement a single 35 ml blood sample will be taken from which DNA will be extracted and electrolytes measured. In addition a single urine sample of up to 100ml followed by a 24 hour collection of urine will be collected at that time and may be repeated when convalescent either towards the end of the hospital stay or after discharge together with a single repeat blood test for electrolyte measurement.

In primary care, collaborating practises will identify patients with contemporaneous hyponatraemia and thiazide prescriptions from their computerised records without disclosure of personal details to the researcher. The General Practitioner will write to the patients and controls explaining the purpose of the research and the request for a single 35ml blood sample. The patient will be invited to respond to the GP with any further questions and arrange an appointment for the practise to acquire written consent and to take a blood sample. If it is not possible for the patient to attend the surgery then the option of either a home visit to take the sample or a saliva collection will be offered. In addition a urine sample of up to 100ml sample and 24 hour collection of urine will be requested. If the patient is hyponatraemic at the time of the first blood and urine sample then a single repeat measurement may be made when convalescent.

In both primary and secondary care it will be made clear that standard treatment will be entirely unaffected either by participation in the study or by declining to participate in the study.

If needed, the usual hospital interpreter and translator services will be available to assist with discussion of the trial, the participant information sheets and consent forms, but the consent forms and information sheets will not be available languages other than English due to the very limited funding for this project. It will be explained to the potential participant that that entry into the trial is entirely voluntary and that their treatment and care will not be affected by their decision. It will also be explained that they can withdraw at any time but attempts will be made to avoid this occurrence. In the event of their withdrawal it

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

will be explained that their data collected so far cannot be erased and we will seek consent to use the data in the final analyses where appropriate.

Inclusion criteria

-Age 18-100 years
-Capacity to give informed consent
-Taking a thiazide or thiazide-related diuretic unless in the non-thiazide control group, or **taking an SSRI or anti-convulsant in the non-thiazide drug induced hyponatraemia control group**
-Blood sodium concentration of equal to or less than 130 mM for cases and sodium between 135 mM and 145 mM for controls

Exclusion criteria

-Patients who lack capacity to consent
-Those who have an alternative medical cause (other than the thiazide) for their hyponatraemia including but not limited to:
-Cirrhosis or other liver failure
-Severe heart failure
-Severe renal failure (eGFR < 30 ml/ minute) or nephrotic syndrome
-Pregnancy
-Untreated endocrine diseases including but not limited to hypothyroidism, hypoadrenalisism and hypopotuotarism)
-Syndrome of Inappropriate Antidiuretic secretion
-Hyperglycaemia (glucose greater than 13.9 mM)

Informed consent

All participants will provide written informed consent and shall be over eighteen years of age. The Informed Consent Form will be signed and dated by the participant before they enter the trial. The Investigator will explain the details of the trial and provide a Participant Information Sheet, ensuring that the participant has sufficient time to consider participating or not. The Investigator will answer any questions that the participant has concerning study participation.

Informed consent will be collected from each participant before they undergo any interventions (including physical examination and history taking) related to the study. One copy of this will be kept by the participant, one will be kept by the Investigator, and a third will be retained in the patient's hospital records.

Should there be any subsequent amendment to the final protocol, which might affect a participant's participation in the trial, continuing consent will be obtained using an amended Consent form which will be signed by the participant.

TRIAL / STUDY TREATMENT AND REGIMEN

Participants shall be contacted by a member of their usual medical team in hospital or General Practitioner in primary care and invited to participate in the study after reading the patient information sheet and having time to ask questions.

If willing the patient shall be asked to sign a consent form and a member of the research team will contact them to take a history of medical circumstances relevant to the study such as previous and ongoing medical problems and medication history.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

If the patient is suitable for inclusion in the study a single venous blood sample (35 ml blood) will be taken by a doctor, nurse or phlebotomist who is fully trained with extensive experience in this technique according to standard methods used in routine clinical care within the NHS. The person who performs the venepuncture shall hold a substantive or honorary contract with the NHS and perform venepuncture as a routine part of their clinical work. A second single 25 ml blood sample shall be taken when convalescent towards the end of the hospital stay or shortly following discharge. For outpatients the urine tests will be at the time of blood sampling and then if necessary repeated when they are recovered from the hyponatraemia several weeks later i.e. at their next clinic visit.

The blood test may be mildly uncomfortable and sometimes results in a small bruise which shall be minimised by the application of pressure at the site of venepuncture as per standard clinical practise in the NHS. Where possible, as these patients will be undergoing venepuncture as part of their normal clinical care, we shall offer to collect blood at the same time as their next clinical sample if desired by the patient.

For patients in primary care unable to attend the GP practise a saliva collection container will be posted to them in place of the blood tests to take DNA.

In addition a urine sample of up to 100ml followed by a 24 hour collection of urine will be collected both in hospital and repeated when convalescent either towards the end of the hospital stay or after discharge. The donation of urine into a plain tube or bottle does not carry any risks to the patient.

Sample size and justification

Since the sample size shall depend on the effect size of an unknown number of genetic determinants of this phenotype, details sample size calculations are not possible.

However based on similar studies in other genetically determined adverse drug effects, 300 cases and 300 controls is likely to be adequate.

This study shall have an 80% power to detect with 95% confidence an effect size of 4.3-6.9 assuming a minor allele frequency of between 25%.

ADVERSE EVENTS

Any adverse events as a result of venepuncture will be dealt with in accordance with NHS procedures. The occurrence of any other adverse as a result of participation within this study is not expected and no adverse event data will be collected.

TRANSPORT AND STORAGE OF THE TISSUES

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

Samples will be stored in linked anonymised format at the Respiratory Medicine Division at Queen's Medical Centre and the Clinical Pharmacology Department at Cambridge University Hospitals NHS Trust. Samples will be labelled using a combination of initials and participant number to permit accurate linkage to clinical data.

For smaller participating recruitment centres, including but not limited to North Devon District Hospital, temporary storage of acute samples until the time of collection of convalescent samples shall occur. Upon completion of sample collection, shipment to the Universities of Nottingham or Cambridge shall occur.

Blood samples will be stored in aliquots, at either -20 or -80 degrees.

Blood and urine samples from other centres shall be transported on ice to the Respiratory Medicine Division at Queen's Medical Centre, Nottingham.

The saliva collection container shall be returned by post to the Respiratory Medicine Division at Queen's Medical Centre, Nottingham.

The master database will be held by Professor I.P. Hall and Dr Mark Glover in a password encrypted file.

The analysis of samples will take place at the University of Nottingham within the Division of Respiratory Medicine.

Samples will be processed and transferred from the Division of Respiratory Medicine to any respective collaborators in batch shipments by courier as frequently as required. All shipments will contain a complete inventory of all samples, along with the name of the person responsible for sending the samples. Once analysis has taken place, samples will be transported back to the Division of Respiratory Medicine at the University of Nottingham and stored within the Research Tissue Bank for future research (DI Prof Jim Lowe-Licence Number 12265) if participants are agreeable and sign the optional clause on the consent form.

Where participants do not agree to the future use of the samples they will be destroyed in accordance with the Human Tissue Act, 2006.

LABORATORY ANALYSES

DNA shall be extracted from blood and saliva collection samples using standard magnetic bead technology. Technologies to analyse DNA such as targeted resequencing and genome wide association studies are constantly evolving and so detailed description is certain to be outdated by the time of performance.

Genetic data attributable to a single individual will be stored on secure university computer(s) and shall be labelled in a linked- or pseudo-anonymised format. Specifically each individual's genetic data shall be contained in a file labelled with initials and participant number.

Western blotting of the 100ml urine sample will be undertaken within the department using a standard protocol.

Analysis of blood and urinary electrolytes will be performed by the biochemistry department at Nottingham University Hospitals NHS Trust using standard techniques employed in routine clinical care.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

Determination of endogenous lithium clearance by measurement of lithium concentration in the blood and urine shall be performed by inductively coupled mass spectrometry at Imperial College Healthcare NHS Trust, London.

STATISTICS

Statistical analysis

Standard statistical techniques for genome wide association and variant calling shall be used and analysis shall be subject to peer review during manuscript submission.

Analysis of differences in endogenous sodium and lithium clearance, 24 hour urinary electrolyte and urinary sodium transporter profile between patients with TIH and controls will be by one way ANOVA with Bonferroni correction.

Primary Outcome Measure

Genetic association between subjects with thiazide-induced hyponatraemia and those without.

ETHICAL AND REGULATORY ASPECTS

Ethical Issues

The study will not be initiated before the protocol, informed consent forms and participant information sheets have received approval / favourable opinion from a Research Ethics Committee (REC). Should a protocol amendment be made that requires REC approval, the changes in the protocol will not be instituted until the amendment and revised informed consent forms and participant and GP information sheets (if appropriate) have been reviewed and received approval / favourable opinion from the REC. A protocol amendment intended to eliminate an apparent immediate hazard to participants or researchers may be implemented immediately providing that the REC is notified as soon as possible and an approval is requested. Minor protocol amendments only for logistical or administrative changes may be implemented immediately; and the REC will be informed.

The study will be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki, 1996; the principles of Good Clinical Practice and Good Laboratory Practice, the DH Research Governance Framework, 2nd ed, 2005 and in accordance with the Human Tissue Act, 2004.

Informed Consent and Participant Information

The process for obtaining participant informed consent will be in accordance with the REC guidance, and Good Clinical Practice (GCP) and any other regulatory requirements that might be introduced. The investigator or their nominee and the participant or other legally authorised representative shall both sign and date the Consent Form before the person can participate in the study.

The participant will receive a copy of the signed and dated forms and the original will be retained in the Study Master File. A second copy will be filed in the participant's medical notes and a signed and dated note made in the notes that informed consent was obtained for the study.

The decision regarding participation in the study is entirely voluntary. The investigator or their nominee shall emphasize to them that consent regarding study participation may be

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

withdrawn at any time without penalty or affecting the quality or quantity of their future medical care, or loss of benefits to which the participant is otherwise entitled. No study-specific interventions will be done before informed consent has been obtained.

If the Consent Form is amended during the study, the investigator shall follow all applicable regulatory requirements pertaining to approval of the amended Informed Consent Form by the REC and use of the amended form (including for ongoing participants).

Withdrawal

The investigator will explain that participation in the study is entirely voluntary and that the participant will retain the right to withdraw at any time without prejudice by simply informing the investigator. Should samples and data already have been obtained we will inform the participant that we would still like to use these samples and data and this will be indicated in the information sheet and consent form. Remaining tissue samples will be destroyed if the participant so wishes.

RECORDS

Source data / documents

Source documents shall be filed at the investigator's site and may include but are not limited to, consent forms, biological data, laboratory notebooks, laboratory results and theatre records. Only study staff shall have access to study documentation other than the regulatory requirements listed below.

Direct access will be permitted when required to all source documents and other study documentation e.g. signed consent forms, for the purpose of study monitoring and audit and other lawful regulatory inspection by the Chief Investigator, Sponsor's designee and inspection by relevant regulatory authorities (e.g., DH, Human Tissue Authority and the REC).

Sample Labelling

Each participant will be assigned a participant number for use on the samples, consent forms and other study documents and the electronic database.

Study documentation will be treated as confidential and held securely in accordance with regulations. The investigator will make a separate confidential record of the participant's name, date of birth, local hospital number or NHS number, and Participant Trial Number (the Trial Recruitment Log), to permit identification of all participants enrolled in the trial, in accordance with regulatory requirements and for follow-up as required

Study documentation shall be restricted to those personnel approved by the Chief or local Principal Investigator and recorded on the 'Trial Delegation Log.'

All paper forms shall be filled in using black ballpoint pen. Errors shall be lined out but not obliterated by using correction fluid and the correction inserted, initialled and dated.

The Chief or local Principal Investigator shall sign a declaration ensuring accuracy of data recorded in the CRF.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

Source documents

Source documents shall be filed at the investigator's site and may include but are not limited to, consent forms, current medical records, laboratory results and records. A CRF may also completely serve as its own source data. Only trial staff as listed on the Delegation Log shall have access to trial documentation other than the regulatory requirements listed below.

Direct access to source data / documents

Study documents, including progress notes and copies of laboratory and medical test results shall made be available at all times for review by the Chief Investigator, Sponsor's designee and inspection by relevant regulatory authorities.

Data Protection

All study staff and investigators will endeavour to protect the study participants' rights to privacy and informed consent, and will adhere to the Data Protection Act, 1998. Only the minimum required information for the purposes of the study shall be collected. Documents will be held securely, in a locked room, or locked cupboard or cabinet. Access to the information will be limited to the study staff and investigators and relevant regulatory authorities (see above). Computer held data including the study database will be held securely and password protected. All data will be stored on a secure dedicated web server. Access will be restricted by user identifiers and passwords (encrypted using a one way encryption method).
Information about the study in the participant's medical records / hospital notes will be treated confidentially in the same way as all other confidential medical information.

QUALITY ASSURANCE & AUDIT

INSURANCE AND INDEMNITY

Insurance and indemnity for study participants and NHS staff is covered within the NHS Indemnity Arrangements for clinical negligence claims in the NHS, issued under cover of HSG (96)48. There are no special compensation arrangements, but study participants may have recourse through the NHS complaints procedures.

The University of Nottingham has taken out an insurance policy to provide indemnity in the event of a successful litigious claim for proven non-negligent harm.

STUDY CONDUCT

Study conduct will be subject to systems audit of the study files for inclusion of essential documents; permissions to conduct the study; CVs of study staff and training received; local document control procedures; consent procedures and recruitment logs; adherence

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

to procedures defined in the protocol (e.g. inclusion / exclusion criteria, timeliness of visits); and equipment calibration logs.

STUDY DATA

Monitoring of study data shall include confirmation of informed consent; source data verification; data storage and data transfer procedures; local quality control checks and procedures, back-up and disaster recovery of any local databases and validation of data manipulation. The Chief Investigator, or where required, a nominated designee, shall carry out monitoring of study data as an ongoing activity.

Data entries will be verified by inspection against the source data. A sample (10% or as per the study risk assessment) will be checked on a regular basis for verification of all entries made. In addition the subsequent capture of the data on any study databases will be checked. Where corrections are required these will carry a full audit trail and justification.
Study data and evidence of monitoring and systems audits will be made available for inspection by the regulatory authority as required.

RECORD RETENTION AND ARCHIVING

In compliance with the DH Research Governance Framework guidelines, the Human Tissue Act and in accordance with the University of Nottingham Code of Research Conduct and Research Ethics, the Chief Investigator will maintain all records and documents of the study. These will be retained for at least 7 years or for longer if required. If the responsible investigator is no longer able to maintain the study records, a second person will be nominated to take over this responsibility.
The study documents held by the Chief Investigator on behalf of the Sponsor shall be finally archived at secure archive facilities at the University of Nottingham. This archive shall include all study databases and associated meta-data encryption codes.

DISCONTINUATION OF THE STUDY BY THE SPONSOR

The Sponsor reserves the right to discontinue this study at any time for failure to meet expected enrolment goals, for safety or any other administrative reasons.

STATEMENT OF CONFIDENTIALITY

Individual participant medical information obtained as a result of this study is considered confidential and disclosure to third parties is prohibited with the exceptions noted above. Participant confidentiality will be further ensured by utilising identification code numbers to correspond to study data in the computer files.

Data generated as a result of this study will be available for inspection on request by the Sponsor, the REC and representatives of the Human Tissue Authority.

PUBLICATION POLICY

The results of genetic analysis of samples in this study shall be submitted for publication in peer reviewed journals and presentation at national and international meetings. At no stage will personal identifiable information be made public or form any part of data submitted for publication or presentation.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

STUDY FINANCES

Funding source

This study is funded by research grants from the Medical Research Council and Nottingham University Hospitals Charity.

Participant stipends and payments

Participants will not be paid to participate in the study. Travel expenses will not be offered for any hospital visits in excess of usual care.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

REFERENCES

1. Williams B, Poulter NR, Brown MJ, Davis M, McInnes GT, Potter JF, Sever PS, McInnes GT, Thom S. Guidelines for management of hypertension: report of the fourth working party of the British Hypertension Society, 2004 - BHS IV. *J Hum Hypertens.* 2004;**18:**139-185.

2. National Institute of Clinical Excellence (NICE). CG34 Hypertension: full guideline (new recommendations and the evidence they are based on). http://guidance.nice.org.uk/CG34/Guidance/pdf/English. 2006.

3. The ALLHAT officers and coordinators for the ALLHAT collaborative research group. Major outcomes in high-risk hypertensive patients randomized to angiotensin converting enzyme inhibitor or calcium channel blocker vs diuretic. *JAMA* 2002;**288:**2981-2997.

4. Glover M, Zuber Am and O'Shaughnessy KM. Hypertension, Dietary Salt Intake, and the Role of the Thiazide-Sensitive Sodium Chloride Transporter NCCT. *Cardiovascular Therapeutics* 2010. E-pub ahead of print.

5. Clayton JA, Rodgers S, Blackey J, Avery A, and Hall IP. Thiazide diuretic prescription and electrolyte abnormalities in primary care. *Br J Clin Pharmacol* 2005;**61:**87-95.

6. Roe PF.Hyponatraemia and diuretics. *Lancet* 1975;**1:**1146-1147.

7. Byatt CM, Millard PH, Levin GE. Diuretics and electrolyte disturbances in 1000 consecutive geriatric admissions. *R J Soc Med* 1990;**83:**704-708.

8. Clayton JA, Le Jeune IR, and Hall IP. Severe hyponatraemia in medical in-patients: aetiology, assessment and outcome. *Q J Med* 2006;**99:**505-511.

9. Ashfar N, Locksley R, Arieff AI. Neurological manifestations and morbidity of hyponatraemia:correlation with brain water and electrolytes. *Medicine (Baltimore)* 1976;**55:**121-129.

10. Leonetti G, Rapelli A, Salvetti A, Scapellato L. Long-term effects of indapamide: final results of a two year Italian multicentre study in systemic hypertension. *Am J Cardiol* 1990;**65:**67H-71H.

11. Elliott WJ, Weber RR, Murphy MB. A double-blind, randomized, placebo-controlled comparison of the metabolic effects of hydrochlorothiazide and indapamide. *J Clin Pharmacol* 1991;**31**:751-757.

12. Chow KM, Szeto CC, Wong TY, Leung CB, Li PK. Risk factors for thiazide-induced hyponatraemia. *Q J Med* 2003;**96:**911-917.

13. Ashraf N, Locksley R, Arieff AI. Thiazide-induced hyponatraemia associated with death or neurologic damage in outpatients. *Am J Med* 1981;**70:**1163-1168.

14. Sharabi Y, Illan R, Kamari T, Cohen H, Nadler M, Messerli FH, Grossman E. Diuretic induced hyponatraemia in elderly hypertensive women. *J Hum Hypertens.* 2002;**16:**631-635.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

15. Abramow M, Cogan E. Clinical aspects and pathophysiology of diuretic-induced hyponatraemia. *Adv Nephrol Necker Hosp* 1984;**13:**1-28.

16. Fidler HM, Goldman J, Bielawska CA, Rai GS, Hoffrband BI. A study of plasma sodium levels in elderly people taking amiloride or triamterine in combination with hydrochlorothiazide. *Postgrad Med J* 1993;**69:**797-799.

17. Clark BA, Shannon RP, Rosa RM and Epstein FH. Increased susceptibility to Thiazide induced Hyponatraemia in the Elderly. *J Am Soc Nephrol* 1994;5:1106-1111.

18. Ashouri OS. Severe diuretic-induced hyponatraemia in the elderly. *Arch Int Med* 1986;**146:**1355-1357.

19. Sonnenblick M, Friedlander Y, Rosin AJ. Diuretic-induced severe hyponatraemia. Review and analysis of 129 reported patients. *Chest* 1993;**103:**601-606.

20. Renneboog B, Musch W, Vandemergel X, Manto MU, Decaux G. Mild chronic hyponatraemia is associated with falls, unsteadiness and attention deficits. *Am J Med* 2006;**119:**71.e1-8.

21. Kengne FG, Andres C, Sattar L, Melot C, Decaux G. Mild hyponatraemia and risk of fracture in the ambulatory elderly. *Q J Med* 2008;**101:**583-588.

22. Adrogue HJ, Madias NE. Hyponatraemia. *N Eng J Med* 2000;**342:**1581-1589.

23. Kumar S, Berl T. Sodium. *Lancet* 1998;**352:**228.

24. Spital A. Diuretic-induced hyponatraemia. *Am J Neprol* 1999;**19:**447-452.

25. Fuisz RE, Lauler DP, Cohen P. Diuretic-induced hyponatraemia and sustained antidiuresis. *Am J Med* 1962;**33:**783-791.

26. Fichman MP, Vorherr H, Kleeman CR, Telfer N. Diuretic-induced hyponatraemia. *Ann Int Med* 1971;**75:**853-863.

27. Hamburger S, Koprivica B, Ellerbeck E, Covinsky JO. Thiazide-induced syndrome of inappropriate secretion of antidiuretic hormone. *JAMA* 1981;**246:**1235-1236.

28. Mann SJ. The silent epidemic of thiazide-induced hyponatraemia. *J Clin Hypertens* 2008;**10:**477-484.

29. Berl T. Treating hyponatraemia. damed if we do and damned if we don't. *Kidney Int* 1990;**37:**1006-1018.

30. Adrogue HJ, Madias NE. Aiding fluid prescription for the dysnatremias. *Intemsive Care Med* 1997;**23:**309-316.

31. Sterns RH. The treatment of hyponatraemia: first, do no harm. *Am J Med* 1990;**88:**557-560.

32. Worthley LI, Thomas PD. Treatment of hyponatraemia seizures with intravenous 29.2% saline. *BMJ (Clin Res Ed)* 1986;**292:**168-170.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

33. Sarnaik AP, Meert K, Hackbarth R, Fleischmann L. Management of hyponatraemia seizures in children with hypertonic saline: a safe and effective strategy. *Crit Care Med* 1991;**19:**758-762.

34. Friedman E, Shadel M, Halkin H, and Farfel Z.Thiazide-induced hyponatremia. Reproducibility by single dose rechallenge and an analysis of pathogenesis. *Ann Int Med* 1989;**110:**24-30.

35. Bobulescu IA, Moe W. Luminal Na(+)/H(+) exchange in the proximal tubule. *Pflugers arch.* 2009;**458:**5-21.

36. Bachmann S, Bostanjoglo M, Schmitt R, Ellison DH. Sodium-transport-related proteins in the mamalian distal nephron - distribution, ontogeny and functional aspects. *Anat Embryol (Berl)* 1999;**200:**447-468.

37. Simon BD, Karet FE, Rodriguez-Soriano J, Hamdan JH, DiPietro A, Trachtman H, Sanjad SA, Lifton RP. Genetic heterogeneity of Bartter's syndrome revealed by mutation in the K⁺ channel, ROMK. *Nat Genet.* 1996;**14:**152-156.

38. Simon BD, Karet FE, Hamdan JM, DiPietro A, Sanjad SA, Lifton RP. Bartter's syndrome, hypokalaemic alkalosis with hypercalciuria, is caused by mutations in the Na-K-Cl cotransporter NKCC2. *Nat Genet.* 1996;**13:**183-188.

39. Simon DB, Nelson-Williams C, Bia MJ, Ellison DH, Karet FE, Molina AM, Vaara I, Iwata F, Cushner HM, Koolen M, Lifton RP. Gitelman's varient of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl transporter. *Nat Genet.* 1996;**12:**24-30.

40. Simon BD, Bindra RS, Mansfield TA, Nelson-Williams C, Mendonca E, Stone R, Schurman S, Nayir A, Alpay H, Bakkaloglu A, Rodriguez-Soriano J, Morales JM, Sanjad SA, Taylor CM, Pilz D, Brem A, Trachtman H, Griswold W, Richard GA, John E, Lifton RP. Mutation in the basolateral Chloride channel CLCNKB, cause Bartter syndrome type III. *Nat Genet.* 1997;**17:**171-178.

41. Gitelman HJ, Graham JB, Welt LG. A new familial disorder characterised by hypokalaemia and hypomagnesaemia. *Trans Assoc Am Physicians* 1966;**79:**221-235.

42. Gordon R, Geddes R, Pawsey C, O'Halloran W. Hypertension and severe hyperkalaemia associated with suppression of renin and aldosterone and completely reversed by dietary sodium restriction. *Aust Ann Med* 1970;**4:**287-294.

43. Disse-Nicodeme S, Achard J, Desitter I, Hout A, Fournier A, Corvol P, Jeunemaitre X. A new locus on chromosome 12p 13.3 for Pseudohypoaldosteronism Type II, an autosomal dominant form of hypertension. *Am.J.Hum.Genet.* 2000;**67:**302-310.

44. Mansfield TA, Simon DB, Farfel Z, Bia M, Tucci JR, Lebel M, Gutkin M, Vialettes B, Christofilis MA, Kauppinen-Makelin R, Mayan H, Risch N, Lifton RP.Multilocus linkage of familial hyperkalaemia and hypertension, familial yperaldosteronism type II to chromosomes 1q31-42 and 17p11-21. *Nat Genet.* 1997;**16:**202-205.

45. Liddle GW, Bledsoe T, Coppage WS Jr. A familial renal disorder simulating primary aldosteronism but with negligible aldosterone secretion. *Trans Assoc Am Physicians* 1963;**76:**199-213.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

46. Chang S, Grunder S, Hanukoglu A, Rösler A, Mathew PM, Hanukoglu I, Schild L, Lu Y, Shimkets RA, Nelson-Williams C, Rossier BC, Lifton RP. Mutations in subunits of the epithelial sodium channel cause salt wasting with hyperkalaemic acidosis,Pseudohypoaldosteronism type I. *Nat Genet.* 1996;**12:**248-253.

47. Geller D, Farahi A, Pinkerton N, Fradley M, Miritz M, Spitzer A, Meinke G, Tsai F, Sigler P, Lifton R. Activating mineralocorticoid receptor mutation in hypertension exacerbated by pregnancy. *Science* 2000;**289:**119-123.

48. Sartorato P, Lapeyraque A-L, Armanini D, Kuhnle U, Khaldi Y, Solomon R, Abadie V, Di Battista E, Naselli A, Racine A, Bosio M, Caprio M, Puolet-Young V, Chabrolle J-P, Niaudet P, De Gennes C, Lecornec M-H, Poisson E, Fusco A, Loli P, Lombes M, Zennaro M-C. Different inactivating mutations of the minerolocorticoid receptor in fourteen families affected by Type I Pseudohypoaldosteronism. *J Clin Endocrinol Metab.* 2003;**88:**2508-2517.

49. Preston GM, Carrol TP, Guggino WB, Agre P. Appearance of water channels in *Xenopus* Oocytes expressing red cell CHIP28 protein. *Science* 1992;**256:**385-387.

50. Litman T, Sogaard R and Zeuthen T. Ammonia and urea permeability of mammalian aquaporins. *Hanb Exp Pharmacol* 2009;**190:**327-358.

51. Nejsum LN. The renal plumbing system: aquarorin water channels. *Cell Mol Life Sci* 2005;**62:**1692-1706.

52. Nielsen S, Smith BL, Christensen EI, Agre P. Distribution of the aquaporin CHIP in secretory and resorptive epithelia and capiliary endothelia. *Proc Natl Acad Sci USA* 1993;**90:**7275-7279.

53. Robben JH, Knoers NV, Deen PM. Cell biological aspects of the vasopressin type-2 receptor and aquaporin 2 water channel in nephrogenic diabetes insipidus. *Am J Physiol Renal Physiol* 2006;**291:**F257-F270.

54. Bichet DG. Lithium, cyclic AMP signaling, A-Kinase Anchoring proteins, and aquaporin 2. *J Am Soc Nephrol* 2006;**17:**920-922.

55. Sands JM, Bichet DG. Nephrogenic diabetes incipidus. *Ann Int Med* 2006;**144:**186-194.

56. Glover M, O'Shaughnessy KM. STK39 and WNK Kinases - a new target for blood pressure treatment? *Curr Opin Nephrol Hypertens.* 2011;**20:**16-22.

57. Subramanya AR, Liu J, Ellison DH, Wade JB, Welling PA. WNK4 diverts the thiazide-sensitive NaCl cotransporter to the lysosome and stimulates AP-3 interaction. *J.Biol.Chem.* 2009;**287:**18471-18480.

58. Richardson C, Rafiqi FH, Karlsson HKR, Molelek N, Vandewalle A, Campbell DG, Morrice NA, Allessi DR. Activation of the thaizide-sensitive Na-Cl cotransporter by the WNK-regulated kinases SPAK and OSR1. *J Cell Sci* 2008;**121:**675-684.

59. Glover M, Mercier Zuber A, O'Shaughnessy KM. Renal and Brain Isoforms of WNK3 have opposite effects on NCCT. *J Am Soc Nephrol* 2009;**20:**1314-1322.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

60. Lin S-H, Shiang J-C, Huang C-C, Yang S-S, Hsu Y-J, Cheng C-J. Phenotype and Genotype Analysis in Chinese Patients with Gitelman's Syndrome. *J Clin Endocrinol Metab.* 2005;**90:**2500-2507.

61. Wang Y, O'Connell JR, McArdle PF, Wade JB, Dorff SE, Shah SJ, Shi X, Pan L, Rampersaud E, Shen H, Kim JD, Subramanya AR, Steinle NI, Parsa A, Ober CC, Welling PA, Chakravarti A, Weder AB, Cooper RS, Mitchell BD, Shuldiner AR, Chang YC. Whole-genome association study identifies STK39 as a hypertension susceptibility gene. *Proc Natl Acad Sci USA* 2009;**106:**226-231.

62. Rafiqi FH, Mercier Zuber A, Glover M, Richardson C, Fleming S, Jovanovic S, Jovanovic A, O'Shaughnessy KM, Alessi DR. Role of the WNK-activated SPAK kinase in regulating blood pressure. *EMBO Mol Med* 2010;**2:**1-13.

63. Clayton JA. The pharmacoepidimiology of hyponatraemia. University of Nottingham.2006;Thesis/Dissertation.

64. Gagnon KBE, England R, Diehl L, Delpire E. Apoptosis-associated tyrosine kinase scaffolding of protein phosphatase 1 and SPAK reveals a novel pathway for Na-K-Cl cotransporter regulation. *Am J Physiol.Cell Physiol.* 2007;**292:**C1809-C1815.

65. Glover M, Zuber AM, Figg N, O'Shaughnessy KM. The activity of the thiazide-sensitive Na+–Cl–cotransporter is regulated by protein phosphatase, PP4. *Can J Physiol Pharmacol* 2010; **88:**986-995.

66. Scholl UI, Choi M, Liu T, Raemaekers VT, Hausler MG, Grimmer J, Tobe SW, Farhi A, Nelson-Williams C, Lifton RP. Seizures, sensorineural deafness, ataxia, mental retardation, and electrolyte imbalance (SeSAME syndrome) caused by muttaions in *KCNJ10. Proc Natl Acad Sci USA* 2009;**106:**5842-5847.

67. Dimke H, San Cristobal P, de Graaf M, Lenders JW, Deinum J, Hoenderop JG, Bindels R. Gamma adducin stimulates the thiazide-sensitive NaCl cotransporter. *J Am Soc Nephrol* 2011;**22:**e-pub ahead of print.

68. San Cristobal P, Pacheco-Alvarez D, Richardson C, Ring AM, Vazquez N, Rafiq FH, Chari D, Kahle KT, Leng Q, Bobadilla NA, Hebert SC, Lifton RP, Gamba G. Angiotensin II signaling increases activity of the renal NaCl cotransporter through a WNK4-SPAK dependent pathway. *Proc Natl Acad Sci USA* 2009;**106:**4384-4389.

69. Magaldi AJ. New insights into the paradoxical effect of thiazides in diabetes insipidus therapy. *Nephrol Dial Transplant.* 2000;**15:**1903-1905.

70. Cesar KR, Magaldi AJ.Thiazide induces water absorption in the inner medullary collecting duct of normal and Brattleboro rats. *Am J Physiol Renal Physiol* 1999, **277:**756-760.

71. Simon DB, Nelson-Williams C, Bia MJ, Ellison DH, Karet FE, Molina AM, Vaara I, 1Iwata F, Cushner HM, Koolen M, Lifton RP. Gitelman's varient of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl transporter. *Nat Genet.* 1996;**12:**24-30.

72. Schepkens H, Stubbe I, Hoeben H, Vanholder R, Lameire N. Severe hyponatraemia and hypouricaemia in Gitelman's syndrome. *Nephrol Dial Transplant.* 2001, **16:**2250-2252.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

73. Weizhen J, Foo JN, O'Roak BJ, Zhao H, Larson MG, Simon BD, Newton-Cheh C, State MW, Levy D, Lifton RP. Rare independent mutations in renal salt handling genes contribute to blood pressure variation. *Nat Genet.* 2008;**5:**592-599.

74. Romeo S, Yin W, Kozlitina J, Pennacchio LA, Boerwinkle E, Hobbs HH, Cohen JC. Rare loss-of-function mutations in *ANGPTL* family members contribute to plasma triglyceride levels in humans. *J Clin Invest.* 2009;**119:**70-79.

75. Uwai Y, Saito H, Hashimoto Y, Inui K-I. Interaction and transport of thiazide diuretics, loop diuretics and acetazolamide via rat renal organic anion transporter rOAT1. *J Pharmacol Expt Ther* 2000;**295:**1261-265.

76. Beermann B, Groschinsky-Grind M, Lindstrom B. Pharmacokinetics of Bendroflumethiazide. *Clin Pharmacol Ther* 1977;**22:**385-388.

77. Delaloy C, Lu J, Hounot AM, Disse-Nicodeme S, Gasc JM, Corvol P, Jeunemaitre X. Multiple promotors in the WNK1 gene: one controls the expression of a kidney-specific kinase-defective isoform. *Mol Cell Biol* 2003;**23:**9208-9221.

78. The SEARCH collaborative group. SLCO1B1 Varients and Statin-Induced-Myopathy- A Genomewide Study. *N Eng J Med* 2008;**359:**789-799.

79. Singer JB, Lewitzky S, Leroy E, Yang F, Zhao X, Klickstein L, Wright TM, Meyer J, Paulding CA. A genome-wide study identifies HLA alleles associated with lumaricoxib-related liver injury. *Nat Genet.* 2010, **42:**711-716.

80. Hautekeete ML, Horsmans Y, Van Waeyenberge C, Demanet C, Henrion J, Verbist L, Brenard R, Sempoux C, Michielsen PP, Yap PS, Rahier J, Geubel AP. HLA association of amoxicillin-clavulanate--induced hepatitis. *Gastroenterology* 1999;**117:**1186.

81. J O'Donohue, K Oien, P Donaldson, J Underhill, M Clare, R MacSween, and P Mills. Co-amoxiclav jaundice: clinical and histological features and HLA class II association. *Gut* 2000;**47:**717-720.

82. Chung WH, Hung SI, Hong HS, Hsih MS, Yang LC, Ho HC, Wu JY, Chen YT. A marker for Stevens-Johnson syndrome. *Nature* 2004;**428:**486.

83. Mallal S *et al.* Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir. *Lancet* 2002;**359:**727-732.

84. Kindmark A, Jawaid A, Harbron CG, Barratt BJ, Bengtsson OF, Andersson TB, Carlsson S, Cederbrant KE, Gibson NJ, Armstrong M, Lagerström-Fermér ME, Dellsén A, Brown EM, Thornton M, Dukes C, Jenkins SC, Firth MA, Harrod GO, Pinel TH, Billing-Clason SM, Cardon LR, March RE. Genome-wide pharmacogenetic investigation of a hepatic adverse event without clinical signs of immunopathology suggests an underlying immune pathogenesis. *Pharmacogenomics J* 2010;**8:**186-195.

85. O'Shaughnessy KM. HapMap, pharmacogenomics, and the goal of personalized prescribing. *Br J Clin Pharmacol* 2006;**61:**783-786.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

9.3.2 Thiazide-Induced Hyponatremia Patient Information Sheet

The University of Nottingham

Nottingham University Hospitals NHS
NHS Trust

Participant Information Sheet
(for patients with non-thiazide drug induced hyponatraemia)

Final Version 1.0 03/October/2017

The Genetics of Salt Imbalance on Thiazide Diuretic Medication

Name of researchers: Professor Ian Hall and Dr Mark Glover

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. We'd suggest this should take about 10 minutes. Talk to others about the study if you wish. Ask us if there is anything that is not clear.

What is the purpose of the study?

The purpose of this study is to try and give us a better understanding of why some individuals get side effects from certain medication and others don't. Thiazide diuretics are a family of tablets commonly prescribed in the treatment of high blood pressure. One of the side effects of treatment with thiazide diuretics is an imbalance of sodium (salt) in the blood. This is usually mild and causes no symptoms but can sometimes be severe and result in serious illness. At present we do not understand why certain individuals develop this side effect and one possibility is that it is due to an inherited (genetic) tendency and it is this that we hope to identify in the study.

Why have I been invited?

You have been invited to take part in this study because the doctors treating you believe you have suffered a low sodium level due to one of the medications you are or were taking. Part of our study involves comparing the characteristics of people who have suffered with a low sodium due to a thiazide diuretic with people who have suffered with a low sodium due to a different drug.

You fall into the second category as you have suffered a low sodium likely as a result of an anti-depressant medication (Selective Serotonin Reuptake Inhibitor, SSRI) or an anti-convulsant medication.

Do I have to take part?

It is up to you to decide to join the study. We will describe the study and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

What will happen to me if I take part?

If you agree to take part in the study we will obtain information about your medical problems and the medication you take from your medical records to confirm that you are eligible to take part.

If you are eligible to take part then we shall take a single sample (33 ml, approximately 7 teaspoons volume), a small urine sample (up to 100ml, approx. 20 teaspoons volume, or whatever you can manage) and a collection of urine over 24 hours. The 24 hour urine collection is made into a large urine bottle and we shall provide specific instructions on how to do this.

A few weeks after you have left hospital, a second blood sample (25 ml, approximately 5 teaspoons volume), small urine sample (up to 100ml, approx. 20 teaspoons volume, or whatever you can manage) and a collection of urine over 24 hours may be requested. This is usually performed at an outpatient clinic in Nottingham but we can visit you in your home if you find it difficult to leave the house.

What will happen to any samples I give?

The blood sample(s) will be analysed for blood salt levels and DNA in your blood will be analysed to look for variations in the genes that control salt and water balance. The urine samples will be analysed for salt and protein levels. Any remaining blood or urine will be kept and stored under our licence.

In addition, if you agree, we may contact you directly in the future about further drug induced hyponatraemia related research projects although you have no obligation to take part in either this or any future study.

Will any genetic tests be done?

DNA in your blood will be analysed to look for variations in the genes that control salt and water balance.

What are the possible advantages, disadvantages and risks of taking part?

We cannot promise the study will help you and you will not receive any individual results of tests performed but the information we get from this study will help improve the treatment of people with blood salt imbalance as a result of taking thiazide diuretics. Our results will be published in scientific journals however it will not be possible to identify individuals who have taken part.

There are no significant disadvantages in taking part other than the time it will take to collect the blood and urine samples and your normal treatment will be entirely unaffected by participation in this research study.

The blood test(s) may be mildly uncomfortable and may leave a small bruise. If desired we can try to arrange for the research blood test(s) to be taken at the same time as any blood tests you may need for your normal hospital care, although this may not always be possible and separate blood tests exclusively for the study may be required.

What are the possible benefits of taking part?

We cannot promise the study will help you, but it is hoped that the information we get from this and future studies will improve understanding of how this side effect occurs and so help to better predict who this may happen to and to design similar medicines with less chance of causing abnormal blood salts.

What will happen if I don't want to carry on with the study?

Your participation is voluntary and you are free to withdraw at any time, without giving any reason, and without your legal rights being affected. If you withdraw then the information collected so far cannot be erased and this information may still be used in the project analysis. Any remaining samples will be destroyed if you wish.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to Dr Mark Glover or another member of the research team who will do their best to answer your questions (telephone 0115 924 9924 and ask for Dr Mark Glover's secretary, or e-mail mark.glover@nottingham.ac.uk).

If you remain unhappy and wish to complain formally, you can do this by contacting the Patient Advice and Liaison Service (PALS), NUH NHS Trust, Freepost NEA 14614, Nottingham, NG7 1BR. Free phone 0800 183 0204 or e-mail pals@nuh.nhs.uk

Involvement of my hospital doctor

If you choose to participate, the doctor(s) looking after you in hospital and your GP will be informed of your involvement in this study and a copy of your consent form filed in your medical records.

Will my taking part in the study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential.

Study data on salt levels in the blood and urine as well as genetic data will be stored in a secure computer database within the university labelled only with your initials and the participant number we will allocate to you. This means that only the research team who have access to the participant number code would be able to identify which data came from which individual participant. The participant number file is kept securely and in confidence with access limited to Professor Hall and nominated members of the research team.

The results of genetic and other tests will not be given to participants, and as such your participation in the study should not affect insurance policies.

We may, if you consent (optional), also use the DNA (genetic material) from your blood samples for further genetic sequencing (analysis) with new genetic techniques as they become available, either within the University of Nottingham or with collaborators at other institutions. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.

What will happen to the results of the research study?

Our results will be published in scientific journals, however it will not be possible to identify individuals who have taken part. The detailed nature of this research and the tests involved means that it may be a year or more before results are published. We are not currently in a position to identify exactly in which scientific journal this will be.

A summary of the study results will be on our departmental website when they are available. The website address is:

http://www.nottingham.ac.uk/medicine/about/respiratorymedicine/index.aspx

Who is organising and funding the research?

This research is organised by clinical academic researchers in the Division of Respiratory Medicine at the University of Nottingham. The study is funded by research grants from the Medical Research Council and Nottingham University Hospitals Charity.

No member of the research team will receive payment over and above their usual employment as clinicians and academics as a result of your participation in this study. Participants will not be paid to participate in the study. Travel expenses will not be offered for any hospital visits in excess of usual care.

Who has reviewed this study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by the Leicester South RECV.

Further information and contact details

Further information is available from Dr Mark Glover, Division of Respiratory Medicine, D Floor, South Block, Queen's Medical Centre, Nottingham, NG7 2UH. E-mail: mark.glover@nottingham.ac.uk, telephone 0115 924 9924

The contact details of the chief investigator are: Professor Ian Hall, Division of Respiratory Medicine, D Floor, South Block, Queen's Medical Centre, Nottingham, NG7 2UH. E-mail: ian.hall@nottingham.ac.uk, telephone 0115 924 9924

We would like to thank you for taking the time to read this document. Please ask us if you have any questions.

9.3.3 Thiazide-Induced Hyponatremia Regulatory Sheet

11 GE003
68666

NHS
National Research Ethics Service

NRES Committee East Midlands - Northampton
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Telephone: 0115 8839368
Facsimile: 0115 8839294

30 AUG 2011
RESEARCH & DEVELOPMENT OFFICE

25 August 2011

Professor Ian P. Hall
Dean of the Medical School
University of Nottingham
Division of Therapeutics and
Molecular Medicine, D Floor, South
Block, Queen's Medical Centre
NG7 2UH

Dear Professor Hall

Study title: **The Pharmacogenetics of Thiazide Induced Hyponatraemia**

REC reference: **11/EM/0233**

Thank you for your letter of 17 August 2011, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Evidence of insurance or indemnity		26 July 2011
Investigator CV		
Letter from Sponsor		15 June 2011
Other: Invitation Letter	Draft 01/Final 1.0 ✓	31 May 2011 ✓
Other: GP Letter	1.0 ✓	17 August 2011 ✓
Participant Consent Form	1.1 ✓	17 August 2011 ✓
Participant Information Sheet: For Hospital Patients	1.1 ✓	17 August 2011 ✓
Participant Information Sheet: For Patients in Primary Care	1.1 ✓	17 August 2011 ✓
Protocol	1.1 ✓	17 August 2011 ✓
REC application	68666/223229/1/435	14 June 2011
Response to Request for Further Information		17 August 2011

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of

changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

11/EM/0233	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

Mr Ken Willis
Chair

Email: lisa.gregory@nottspct.nhs.uk

Enclosures: "After ethical review – guidance for researchers" *SL-AR2*

Copy to: *Dr Mark Glover*
Mr Paul Cartledge
Dr. Maria Koufali, Research and Development

9.3.4 Gordon Syndrome Ethical Protocol

The University of Nottingham

Clinical and genetic characterisation of Gordon syndrome

Final Version 1.3
21 January 2016

Short title:	Clinical and genetic characterisation of Gordon syndrome
Acronym:	GS
NRES reference:	12/EM/0317
Trial Sponsor:	University of Nottingham
Sponsor reference:	12082
Funding Source:	Medical Research Council

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

STUDY PERSONNEL AND CONTACT DETAILS

Sponsor:
Contact name

University of Nottingham
Mr Paul Cartledge
Head of Research Grants and Contracts
Research and Graduate Services
King's Meadow Campus
Lenton Lane
Nottingham
NG7 2NR

Chief investigator:

Dr Mark Glover
Division of Respiratory Medicine,
D Floor, South Block,
Queen's Medical Centre,
Nottingham
NG7 2UH
Phone: +44 (0)115 82 31063
Fax: +44 (0)115 82 31059
Email: mark.glover@nottingham.ac.uk

Co-investigators:

Professor Ian P. Hall
Division of Respiratory Medicine,
D Floor, South Block,
Queen's Medical Centre,
Nottingham
NG7 2UH
Phone: +44 (0)115 82 31063
Fax: +44 (0)115 82 31059
Email: ian.hall@nottingham.ac.uk

Dr K.M. O'Shaughnessy
Clinical Pharmacology Unit,
Box 110,
Addenbrooke's Hospital,
Cambridge
CB2 2QQ
Phone: +44 (0) 1223 762578
Email: kmo22@medschl.cam.ac.uk

Study Coordinating Centre:

Division of Respiratory Medicine,
University of Nottingham,
D Floor, South Block,
Queen's Medical Centre,
Nottingham,
NG7 2UH

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

STUDY SYNOPSIS

Title	Clinical and genetic characterisation of Gordon's syndrome
Acronym	GS
Chief Investigator	Dr M Glover
Study centre(s)	Nottingham University Hospitals NHS Trust Cambridge University Hospitals NHS Foundation Trust
Objectives	1) To determine the genetic basis of Gordon syndrome 2) To detail the clinical phenotype of Gordon syndrome
Number of participants / tissue samples	200
Diagnosis and main criteria for inclusion	Inclusion criteria: 1) Age 18 years and over 2) Have the capacity to give informed consent 3) Have a clinical diagnosis of Gordon syndrome or to be a relative of an individual with Gordon syndrome
Duration of study	Overall: 01 September 2012 – 01 September 2022 Per participant: 5 minutes for consent 2x5 minutes for 2 venepunctures 5 minutes each for two small spot urine samples 2x 24 hour urine collections
Description of interventions	Two venepunctures Two small urine samples Two 24 hour urine collections
Statistical methods	Association testing only

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

Table of contents

STUDY PERSONNEL AND CONTACT DETAILS 2
Sponsor 2
STUDY SYNOPSIS 3
ABBREVIATIONS 5
STUDY BACKGROUND INFORMATION AND RATIONALE 5
Summary 5
STUDY OBJECTIVES AND PURPOSE 8
PURPOSE 8
PRIMARY OBJECTIVE 8
SECONDARY OBJECTIVES 8
STUDY DESIGN 9
STUDY CONFIGURATION 9
Primary endpoint 9
Secondary endpoint 9
TRIAL MANAGEMENT 9910
DURATION OF THE TRIAL / STUDY AND PARTICIPANT INVOLVEMENT 10
End of the Study 10
SELECTION AND WITHDRAWAL OF STUDY PARTICIPANTS 10
Recruitment 10
Eligibility criteria 11
Inclusion criteria 11
Exclusion criteria 11
Informed consent 11
STUDY TREATMENT AND REGIMEN 12
Sample size and justification 12
ADVERSE EVENTS 12
TRANSPORT AND STORAGE OF THE TISSUES 12
LABORATORY ANALYSES 13
STATISTICS 13
Statistical analysis 13
Primary Outcome Measure 14
ETHICAL AND REGULATORY ASPECTS 14
Ethical Issues 14
Informed Consent and Participant Information 14
Withdrawal 14
RECORDS 15
Sample Labelling 15
Source documents 161615
Direct access to source data / documents 16
Data Protection 16
QUALITY ASSURANCE & AUDIT 16
INSURANCE AND INDEMNITY 16
STUDY CONDUCT 16
STUDY DATA 17
RECORD RETENTION AND ARCHIVING 17
DISCONTINUATION OF THE STUDY BY THE SPONSOR 17
STATEMENT OF CONFIDENTIALITY 17
PUBLICATION POLICY 17

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

STUDY FINANCES 18
Funding source 18
Participant stipends and payments 18
REFERENCES 18

ABBREVIATIONS

AATYK Apoptosis-Associated TYrosine Kinase
AC Adenylate Cyclase
ADH AntiDiuretic Hormone
AP PKA anchoring proteins
AQP Aquaporin
AT1R Angiotensin 1 Receptor
ATP Adenosine triphosphate
cAMP cyclic Adenosine MonoPhosphate
CLCNKB Basolateral membrance chloride transporter
COX Cyclo-OXygenase
ER Endoplasmic Reticulum
Gαs G-protein alpha subunit
GWAS Genome Wide Association Study
GS Gordon Syndrome
KCNJ10 ATP-sensitive inward rectifier potassium channel 10
MR Mineralocorticoid Receptor
NCC Thiazide-sensitive sodium chloride transporter
NKCC2 Loop diuretic sensitive sodium transporter
NHE3 Proximal tubule sodium transporter
OAT Organic Anion Transporter,
PCT Proximal Convoluted Tubule
PG ProstaGlandin
PHAII PseudoHypoAldosteronism type 2
PKA Protein kinase A
PP4 Protein Phosphatase 4
ROMK Renal Outer Medullary potassium transporter
SPAK STE-20/SPS1 proline/alanine-rich kinase
TAL Thick Ascending Loop of Henle
TIH Thiazide Induced Hyponatraemia
TGN Trans Golgi Network
V2R Vasopressin 2 receptor
WNK With No Lysine (K) kinase

STUDY BACKGROUND INFORMATION AND RATIONALE

Summary

Renal reabsorption of sodium by the thiazide-sensitive pathway is central to hypertension pathogenesis and yet thiazide diuretics, despite their widespread use, are of limited efficacy and produce side effects commonly.

This study aims to understand thiazide-sensitive sodium trafficking at a molecular level by studying one clinical manifestation of dysregulation of this pathway: Gordon syndrome (GS, a monogenic and thiazide-responsive form of hypertension).

The genetic basis for GS in patients without currently described mutations will be elucidated by whole exome resequencing and phenotype-genotype correlations detailed in collaboration with Professor Gordon. The relevance of identified genes to blood pressure

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

and sodium concentration in the general population will be investigated using large existing study databases and the function of these genes characterised using *in vitro* and *in vivo* models.

The potential benefits of uncovering the molecular mechanisms underlying the regulation of distal nephron sodium and water absorption include the advancement of the design of better tolerated, more efficacious thiazide-like antihypertensive agents.

Background

Hypertension is the most common modifiable cause of cardiovascular disease - the leading cause of mortality worldwide[1,2]. 25% of the UK adult population is hypertensive, and treatment consumes over a billion pounds in NHS drug costs annually[3].

Whilst the treatment of hypertension is both evidence based and cost effective[1], most patients require multiple anti-hypertensive agents, side-effects are commonplace and blood pressure control for many remains poor[4]. Current therapeutic limitations are sadly unsurprising when hypertension pathogenesis still largely defies attempts at explanation in the majority of affected individuals.

The association of hypertension with salt is intriguing however; sodium chloride intake correlates with the prevalence of hypertension even in disparate populations[5], and sodium restriction lowers blood pressure, especially in those already hypertensive[6]. The physiological effects of salt are not simply a function of sodium intake but rather a balance of oral intake vs. excretion into the urine. Regulation of sodium reabsorption from tubular filtrate in the distal nephron has a marked effect on blood pressure and although the amiloride-sensitive Epithelial Sodium (Na) Channel (ENaC) has classically dominated research interest, Na-Cl reabsorption via the thiazide-sensitive sodium transporter, NCC, is at least as important [7]. Thiazide diuretics are potent antihypertensive agents[3] and mimic the effects of loss-of function NCC mutations seen in the hypotensive monogenic syndrome of Gitelman[20]. Moreover, the heritable condition of hyperkalaemic hypertension, Gordon syndrome, results from increased sodium reabsorption via NCC and is effectively cured by thiazide diuretics[8].

Mechanisms of sodium reabsorption

The kidneys filter some 170 litres of plasma daily containing around 23 moles of sodium, of which over 99.5% is reabsorbed by a series of sodium transporters along the nephron (Figure 1). The greatest fraction of filtered sodium (60-70%) is reabsorbed in the proximal convoluted tubule (PCT) by the Na^+/H^+ exchanger NHE3 [9].

A further 20-30% is reabsorbed in the Thick Ascending limb of the Loop of Henle (TAL) by the Na-K-2Cl transporter NKCC2, the target of Loop diuretics such as furosemide [10]. Potassium is recycled across the apical membrane, in a rate limiting manner, by the renal outer medulla potassium channel, ROMK, whereas Cl^- exits the cell via the basolateral membrane through chloride channels and transporters including CLCNKB. Mutations within any of these genes (NKCC2, ROMK, and CLCNKB) leads to Bartter syndrome: type I, II, and III respectively [11,12,13,14]. Bartter syndrome is a salt wasting condition, mimicking treatment with Loop diuretics and is characterized by hypokalemia, metabolic alkalosis, hypercalciuria and a tendency to hypotension.

Of the remaining 10% of sodium reaching the distal nephron, 5-7% is reabsorbed by the thiazide-sensitive Na-Cl cotransporter, NCC in the distal convoluted tubule (DCT). The DCT is the site of two other monogenic diseases. First, loss of-function mutations within NCC lead to Gitelman syndrome [15], which resembles therapy with thiazide diuretics. Second, Gordon syndrome [16] (pseudohypoaldosteronism type 2 or PHAII), the clinical mirror of Gitelman syndrome results from increased NCC membrane expression; it is characterized by hypertension and hyperkalemic acidosis and caused by mutations within

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

one of two members of a novel family of regulatory kinases called the "With No lysine (K)" kinases (WNK1 and WNK4) [17,18].

A final 2-3% of filtered sodium is reabsorbed in the connecting tubule (CNT) and the collecting duct (CD) by the amiloride-sensitive epithelial sodium channel, ENaC. Gain of function mutations in ENaC cause Liddle syndrome, a condition of hypokalaemic alkalosis and low renin hypertension [19]. Conversely loss of function ENaC mutation leads to an autosomal recessive syndrome of salt wasting termed pseudohypoaldosteronism type 1 [20].

ENaC regulation by aldosterone is reflected by syndromes of hyper- and hypo-tension caused by gain- and loss-of function mutations in the mineralocorticoid receptor: the syndrome of activating mineralocorticoid receptor mutation [21] and a mild form of autosomal dominant pseudohypoaldosteronism type 1 respectively [22].

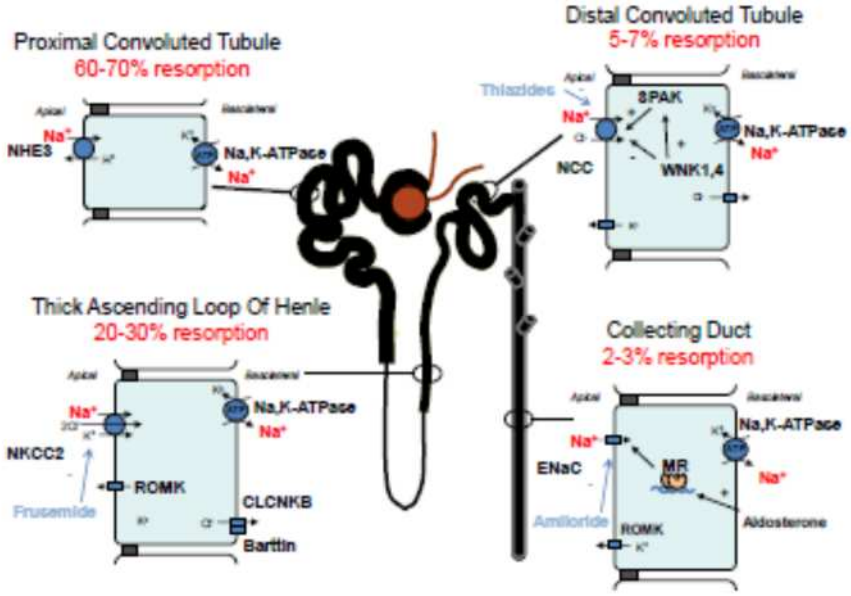


Figure 1
Schematic representation of the nephron showing principal sites of sodium reabsorption. In the proximal convoluted tubule via NHE3, Na^+/H^+ exchanger isoform 3; in the thick ascending Loop of Henle via the Frusemide-sensitive NKCC2, $Na^+/K^+/2Cl^-$ cotransporter; in the distal convoluted tubule by the thiazide-sensitive NCC, Na^+/Cl^- cotransporter and in the collecting duct via the amiloride-sensitive epithelial sodium channel (ENaC). ROMK (renal outer medulla potassium channel), CLCNKB (chloride channel kidney B), SPAK (STE-20/SPS1 proline/alanine-rich kinase), WNKs 1/4 (with no lysine kinases 1/4) and MR (mineralocorticoid receptor) are also shown.

Gordon syndrome (GS, or Pseudohypoaldosteronism type 2)

Gordon syndrome is characterised by hypertension, hyperkalaemic acidosis and exquisite sensitivity to low-dose thiazide diuretics[8]. Inheritance is usually autosomal dominant although recessive and *de novo* occurrence is also described[8,23]. As in Liddle syndrome, significant inter- and intra-pedigree phenotypic variation is observed clinically [23,24]. Causative mutations within one of two members of the novel family of regulatory WNK kinases (WNKs 1&4)[24,25], but not NCC itself[26], have been identified in the limited number of families studied to date. NCC regulation is complex, complicated and incompletely understood; it involves a scaffold of at least a dozen or so interacting proteins centred on a WNK signalling cascade, with the STE20 kinases (SPAK and OSR1) as the 'missing'

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

intermediates that are activated by WNKs and which in turn activate NCC[27,28,29]. We, together with others, distinguished two distinct modes of NCC regulation illustrated in Figure 2.

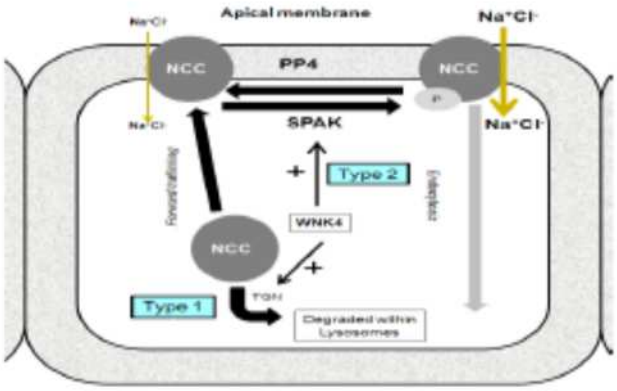


Figure 2: The two principal mechanisms of regulation of the thiazide-sensitive Na+/Cl- cotransporter, NCC.
Type 1 regulation affects forward trafficking of NCC to the apical membrane. At least for wild type WNK4, this appears to be by diversion of NCC through the trans-golgi network (TGN) for degradation in lysosomes.
Type 2 regulation of NCC determines intrinsic transporter kinetics by phosphorylation of key N-terminal serine/ threonine residues such as T58. This phosphorylation is mediated by STE-20/SPS1 proline/alanine-rich kinase (SPAK) which in turn is activated by WNK4. Protein phosphatase 4 (PP4) may counter SPAK in *type 2* regulation by dephosphorylation of NCC.

Synopsis of investigation

We shall undertake targeted resequencing of the four currently identified causative genes in GS (WNK1, WNK4, CUL3 and KLHL3) to determine whether affected individuals possess either a known or novel mutation within these genes. We shall undertake detailed phenotypic measurements of these individuals by means of blood and urine tests.

For GS patients who do not have a mutation in the 4 known genes we shall proceed to whole exome resequencing to identify novel associated genes and mutations.

STUDY OBJECTIVES AND PURPOSE

PURPOSE

This is a case control study.

PRIMARY OBJECTIVE

To establish the genetic basis for Gordon syndrome.

SECONDARY OBJECTIVES

To better characterise the plasma and urinary electrolyte profile of patients with GS relative to their unaffected family members.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

STUDY DESIGN

STUDY CONFIGURATION

The study shall be managed from the University of Nottingham but shall recruit from Nottingham University Hospitals NHS Trust, Cambridge University Hospitals NHS Foundation Trust, and any other clinical genetics service either within or out with the UK who wishes to offer the possibility of establishing a molecular genetics diagnosis to their patients with hyperkalaemic hypertension.

We propose to conduct a targeted resequencing and whole exome study to characterise genetic polymorphisms associated with GS. These genetic approaches have been successful in understanding how genetic variation contributes to blood pressure and cholesterol regulation at a molecular level. It is hoped a similar approach for GS will improve the design of new blood pressure lowering drugs.

We shall also characterise the phenotype of GS by measurement of 24 hour urinary electrolytes, endogenous sodium and lithium clearance and abundance of sodium transporters in the urine in patients and controls.

This research proposal has been developed by Professor Ian Hall (Chair of Therapeutics and Professor of Clinical Pharmacology), Dr Mark Glover (Associate Professor and Honorary Consultant Physician) and Dr Kevin O'Shaughnessy (Senior Lecturer and Honorary Consultant physician).

The research protocol is subject to peer review from the Medical Research Council and British Heart Foundation. We have also consulted patients regarding the design of the study and consent procedures. Patients have been consulted both in hospital who suffer from GS and in hypertension clinic who are taking a thiazide diuretic but who do not have GS.

Recruitment

Timescale

Recruitment will begin in September 2012 or as soon after this date that approval is granted and will continue for 10 years.

Primary endpoint

Collection of blood samples from which DNA shall be extracted.

Secondary endpoint

Genetic analysis to determine associations with GS by means of targeted resequencing and whole exome techniques.

Characterisation of the plasma and urinary electrolyte profile of patients with GS compared to controls.

Characterisation of the urinary sodium transporter profile in patients with GS and controls

TRIAL MANAGEMENT

This research proposal has been developed by Professor Ian Hall (Chair of Therapeutics and Professor of Clinical Pharmacology), Dr Mark Glover (Associate Professor and

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

Honorary Consultant Physician) and Dr Kevin O'Shaughnessy (Senior Lecturer and Honorary Consultant Physician).

All aspects of recruitment, storage and analysis of samples and the resulting genetic association techniques shall be overseen by Professor Hall and Dr Mark Glover with results submitted for publication in peer reviewed journals as well as presented at key national and international scientific meetings.

The Chief Investigator has overall responsibility for the study and shall oversee all study management.

The data custodian will be the Chief Investigator.

DURATION OF THE TRIAL / STUDY AND PARTICIPANT INVOLVEMENT

In hospital the blood sample may be taken after the consent i.e. on the same day unless the patient requests otherwise. Urine samples will be taken contemporaneously on the day of the blood sample and again when convalescent either shortly before discharge from hospital or shortly after discharge from hospital.

The study will run for 10 years. Each participant will be directly involved in the study for the 24 hour period during which blood and urine samples are collected.

End of the Study

The end of the study will be the last blood and urine sample of the last participant.

Laboratory analyses will continue depending on progress at that point and developments in genetic technologies.

SELECTION AND WITHDRAWAL OF STUDY PARTICIPANTS

Recruitment

Participants will be recruited from hypertension or clinical genetics clinics. The initial approach will be from a member of the patient's usual care team (which may include the investigator). Family members will be approached by the local clinical genetics service as per standard NHS clinical genetics contact tracing practise.

The investigator or their nominee, e.g. from the research team or a member of the participant's usual care team, will inform the participant or their nominated representative (other individual or other body with appropriate jurisdiction), of all aspects pertaining to participation in the study.

Following identification informed consent will be sought and if the patient is in agreement a single 33 ml blood sample will be taken from which DNA will be extracted and electrolytes measured. In addition a single urine sample of up to 100ml followed by a 24 hour collection of urine will be collected at that time. The blood and urine samples may be repeated if initial measurements are made before the patient has started thiazide diuretic medication

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

as part of their routine clinical management of hypertension. Therapy will not be altered by participation in this study.

It will be made clear that standard treatment will be entirely unaffected either by participation in the study or by declining to participate in the study.

If needed, the usual hospital interpreter and translator services will be available to assist with discussion of the trial, the participant information sheets and consent forms, but the consent forms and information sheets will not be available languages other than English due to the very limited funding for this project. It will be explained to the potential participant that that entry into the trial is entirely voluntary and that their treatment and care will not be affected by their decision. It will also be explained that they can withdraw at any time but attempts will be made to avoid this occurrence. In the event of their withdrawal it will be explained that their data collected so far cannot be erased and we will seek consent to use the data in the final analyses where appropriate.

Eligibility criteria

Inclusion criteria

-Age 18 years and over
-Capacity to give informed consent
-Clinical diagnosis of Gordon syndrome (hyperkalaemia and hypertension) for cases or a family member of an individual with Gordon syndrome for controls

Exclusion criteria

-Patients who lack capacity to consent
-Those who neither have Gordon syndrome nor a family member with Gordon syndrome

Informed consent

All participants will provide written informed consent and shall be over eighteen years of age. The Informed Consent Form will be signed and dated by the participant before they enter the trial. The Investigator will explain the details of the trial and provide a Participant Information Sheet, ensuring that the participant has sufficient time to consider participating or not. The Investigator will answer any questions that the participant has concerning study participation.

Informed consent will be collected from each participant before they undergo any interventions (including physical examination and history taking) related to the study. One copy of this will be kept by the participant, one will be kept by the Investigator, and a third will be retained in the patient's hospital records.

Should there be any subsequent amendment to the final protocol, which might affect a participant's participation in the trial, continuing consent will be obtained using an amended Consent form which will be signed by the participant.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

STUDY TREATMENT AND REGIMEN

Participants shall be recruited by their usual clinician and invited to participate in the study after reading the patient information sheet and having time to ask questions.

If willing the patient shall be asked to sign a consent form and a member of the research team will contact them to take a history of medical circumstances relevant to the study such as previous and ongoing medical problems and medication history.

If the patient is suitable for inclusion in the study a single venous blood sample (30 ml blood) will be taken by a doctor, nurse or phlebotomist who is fully trained with extensive experience in this technique according to standard methods used in routine clinical care within the NHS. The person who performs the venepuncture shall hold a substantive or honorary contract with the NHS and perform venepuncture as a routine part of their clinical work. A second single 23ml blood sample may be taken if the initial measurement was taken before thiazide diuretics were started as part of routine clinical care.

The blood test may be mildly uncomfortable and sometimes results in a small bruise which shall be minimised by the application of pressure at the site of venepuncture as per standard clinical practise in the NHS. Where possible, as these patients will be undergoing venepuncture as part of their normal clinical care, we shall offer to collect blood at the same time as their next clinical sample if desired by the patient.

In addition a single urine sample of up to 100ml followed by a 24 hour collection of urine will be collected both initially and may be repeated if the initial sample was taken before thiazide diuretic medication was started as part of routine clinical care. The donation of urine into a plain tube or bottle does not carry any risks to the patient.

Sample size and justification

Since the sample size shall depend on the effect size of an unknown number of genetic determinants of this phenotype, detailed sample size calculations are not possible.

Owing to the extreme rarity of Gordon syndrome the numbers of participants will be determined by the numbers of affected individuals who can be identified. This strategy has enjoyed considerable success in recent years using similar numbers of participants in GS and other rare heritable disorders of hypertension and renal sodium retention.

ADVERSE EVENTS

Any adverse events as a result of venepuncture will be dealt with in accordance with NHS procedures. The occurrence of any other adverse as a result of participation within this study is not expected and no adverse event data will be collected.

TRANSPORT AND STORAGE OF THE TISSUES

Samples will be stored in linked anonymised format at the Respiratory Medicine Division at Queen's Medical Centre and the Clinical Pharmacology Department at Cambridge University Hospitals NHS Trust. Samples will be labelled using a combination of initials and participant number to permit accurate linkage to clinical data.

Blood samples will be stored in aliquots, at either -20 or -80 degrees.

Blood and urine samples from other centres shall be transported on ice to the Respiratory Medicine Division at Queen's Medical Centre, Nottingham.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

The master database will be held by Professor I.P. Hall and Dr Mark Glover in a password encrypted file.

The analysis of samples will take place at the University of Nottingham within the Division of Respiratory Medicine.

Samples will be processed and transferred from the Division of Respiratory Medicine to any respective collaborators in batch shipments by courier as frequently as required. All shipments will contain a complete inventory of all samples, along with the name of the person responsible for sending the samples. Once analysis has taken place, samples will be transported back to the Division of Respiratory Medicine at the University of Nottingham and stored within the Research Tissue Bank for future research (DI Prof Jim Lowe- Licence Number 12265) if participants are agreeable and sign the optional clause on the consent form.

Where participants do not agree to the future use of the samples they will be destroyed in accordance with the Human Tissue Act, 2006.

LABORATORY ANALYSES

DNA shall be extracted from blood samples using standard magnetic bead technology. Technologies to analyse DNA such as targeted resequencing and whole exome studies are constantly evolving and so detailed description is certain to be outdated by the time of performance.

Genetic data attributable to a single individual will be stored on secure university computer(s) and shall be labelled in a linked- or pseudo-anonymised format. Specifically each individual's genetic data shall be contained in a file labelled with initials and participant number.

Western blotting of the **100ml** urine sample will be undertaken within the department using a standard protocol.

Analysis of blood and urinary electrolytes will be performed by the biochemistry department at Nottingham University Hospitals NHS Trust using standard techniques employed in routine clinical care.

Determination of endogenous lithium clearance by measurement of lithium concentration in the blood and urine shall be performed by inductively coupled mass spectrometry at Imperial College Healthcare NHS Trust, London.

STATISTICS

Statistical analysis

Standard statistical techniques for genome wide association and variant calling shall be used and analysis shall be subject to peer review during manuscript submission.

Analysis of differences in endogenous sodium and lithium clearance, 24 hour urinary electrolyte and urinary sodium transporter profile between patients with GS and controls will be by one way ANOVA with Bonferroni correction.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

Primary Outcome Measure

Establishment of the genetic basis of Gordon syndrome

ETHICAL AND REGULATORY ASPECTS

Ethical Issues

The study will not be initiated before the protocol, informed consent forms and participant information sheets have received approval / favourable opinion from a Research Ethics Committee (REC). Should a protocol amendment be made that requires REC approval, the changes in the protocol will not be instituted until the amendment and revised informed consent forms and participant information sheets (if appropriate) have been reviewed and received approval / favourable opinion from the REC. A protocol amendment intended to eliminate an apparent immediate hazard to participants or researchers may be implemented immediately providing that the REC is notified as soon as possible and an approval is requested. Minor protocol amendments only for logistical or administrative changes may be implemented immediately; and the REC will be informed.

The study will be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki, 1996; the principles of Good Clinical Practice and Good Laboratory Practice, the DH Research Governance Framework, 2nd ed, 2005 and in accordance with the Human Tissue Act, 2004.

Informed Consent and Participant Information

The process for obtaining participant informed consent will be in accordance with the REC guidance, and Good Clinical Practice (GCP) and any other regulatory requirements that might be introduced. The investigator or their nominee and the participant or other legally authorised representative shall both sign and date the Consent Form before the person can participate in the study.

The participant will receive a copy of the signed and dated forms and the original will be retained in the Study Master File. A second copy will be filed in the participant's medical notes and a signed and dated note made in the notes that informed consent was obtained for the study.

The decision regarding participation in the study is entirely voluntary. The investigator or their nominee shall emphasize to them that consent regarding study participation may be withdrawn at any time without penalty or affecting the quality or quantity of their future medical care, or loss of benefits to which the participant is otherwise entitled. No study-specific interventions will be done before informed consent has been obtained.

If the Consent Form is amended during the study, the investigator shall follow all applicable regulatory requirements pertaining to approval of the amended Informed Consent Form by the REC and use of the amended form (including for ongoing participants).

Withdrawal

The investigator will explain that participation in the study is entirely voluntary and that the participant will retain the right to withdraw at any time without prejudice by simply informing the investigator. Should samples and data already have been obtained we will inform the participant that we would still like to use these samples and data and this will be indicated in the information sheet and consent form. Remaining tissue samples will be destroyed if the participant so wishes.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

RECORDS

Each participant will be assigned a trial identity code numberfor use on CRFs other trial documents and the electronic database. The documents and database will also use their initials.

CRFs will be treated as confidential documents and held securely in accordance with regulations. The investigator will make a separate confidential record of the participant's name, date of birth, local hospital number or NHS number, and Participant Study Number (the Study Recruitment Log), to permit identification of all participants enrolled in the trial, in accordance with regulatory requirements and for follow-up as required

CRFs shall be restricted to those personnel approved by the Chief or local Principal Investigator and recorded on the 'Study Delegation Log.'

All paper forms shall be filled in using black ballpoint pen. Errors shall be lined out but not obliterated by using correction fluid and the correction inserted, initialled and dated.

The Chief or local Principal Investigator shall sign a declaration ensuring accuracy of data recorded in the CRF.

Source documents shall be filed at the investigator's site and may include but are not limited to, consent forms, biological data, laboratory notebooks, and laboratory results. Only study staff shall have access to study documentation other than the regulatory requirements listed below.

Direct access will be permitted when required to all source documents and other study documentation e.g. signed consent forms, for the purpose of study monitoring and audit and other lawful regulatory inspection by the Chief Investigator, Sponsor's designee and inspection by relevant regulatory authorities (e.g., DH, Human Tissue Authority and the REC).

Sample Labelling

Each participant will be assigned a participant number for use on the samples, consent forms and other study documents and the electronic database.

Study documentation will be treated as confidential and held securely in accordance with regulations. The investigator will make a separate confidential record of the participant's name, date of birth, local hospital number or NHS number, and Participant Trial Number (the Trial Recruitment Log), to permit identification of all participants enrolled in the trial, in accordance with regulatory requirements and for follow-up as required

Study documentation shall be restricted to those personnel approved by the Chief or local Principal Investigator and recorded on the 'Trial Delegation Log.'

All paper forms shall be filled in using black ballpoint pen. Errors shall be lined out but not obliterated by using correction fluid and the correction inserted, initialled and dated.

The Chief or local Principal Investigator shall sign a declaration ensuring accuracy of data recorded in the CRF.

Samples for NHS pathology analysis will be labelled in accordance with local NHS procedures.

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

Source documents

Source documents shall be filed at the investigator's site and may include but are not limited to, consent forms, current medical records, laboratory results and records. A CRF may also completely serve as its own source data. Only trial staff as listed on the Delegation Log shall have access to trial documentation other than the regulatory requirements listed below.

Direct access to source data / documents

Study documents, including progress notes and copies of laboratory and medical test results shall made be available at all times for review by the Chief Investigator, Sponsor's designee and inspection by relevant regulatory authorities.

Data Protection

All study staff and investigators will endeavour to protect the study participants' rights to privacy and informed consent, and will adhere to the Data Protection Act, 1998. Only the minimum required information for the purposes of the study shall be collected. Documents will be held securely, in a locked room, or locked cupboard or cabinet. Access to the information will be limited to the study staff and investigators and relevant regulatory authorities (see above). Computer held data including the study database will be held securely and password protected. All data will be stored on a secure dedicated web server. Access will be restricted by user identifiers and passwords (encrypted using a one way encryption method).
Information about the study in the participant's medical records / hospital notes will be treated confidentially in the same way as all other confidential medical information.

QUALITY ASSURANCE & AUDIT

INSURANCE AND INDEMNITY

Insurance and indemnity for study participants and NHS staff is covered within the NHS Indemnity Arrangements for clinical negligence claims in the NHS, issued under cover of HSG (96)48. There are no special compensation arrangements, but study participants may have recourse through the NHS complaints procedures.

The University of Nottingham as research Sponsor indemnifies its staff, research participants and research protocols with both public liability insurance and clinical trials insurance. These policies include provision for indemnity in the event of a successful litigious claim for proven non-negligent harm.

STUDY CONDUCT

Study conduct will be subject to systems audit of the study files for inclusion of essential documents; permissions to conduct the study; CVs of study staff and training received;

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

local document control procedures; consent procedures and recruitment logs; adherence to procedures defined in the protocol (e.g. inclusion / exclusion criteria, timeliness of visits); and equipment calibration logs.

The Chief Investigator, or where required, a nominated designee, shall carry out a site systems audit at least yearly and an audit report shall be made to the Study Sponsor.

STUDY DATA

Monitoring of study data shall include confirmation of informed consent; source data verification; data storage and data transfer procedures; local quality control checks and procedures, back-up and disaster recovery of any local databases and validation of data manipulation. The Chief Investigator, or where required, a nominated designee, shall carry out monitoring of study data as an ongoing activity.

Data entries will be verified by inspection against the source data. A sample (10% or as per the study risk assessment) will be checked on a regular basis for verification of all entries made. In addition the subsequent capture of the data on any study databases will be checked. Where corrections are required these will carry a full audit trail and justification.
Study data and evidence of monitoring and systems audits will be made available for inspection by the regulatory authority as required.

RECORD RETENTION AND ARCHIVING

In compliance with the DH Research Governance Framework guidelines, the Human Tissue Act and in accordance with the University of Nottingham Code of Research Conduct and Research Ethics, the Chief Investigator will maintain all records and documents of the study. These will be retained for at least 7 years or for longer if required. If the responsible investigator is no longer able to maintain the study records, a second person will be nominated to take over this responsibility.
The study documents held by the Chief Investigator on behalf of the Sponsor shall be finally archived at secure archive facilities at the University of Nottingham. This archive shall include all study databases and associated meta-data encryption codes.

DISCONTINUATION OF THE STUDY BY THE SPONSOR

The Sponsor reserves the right to discontinue this study at any time for failure to meet expected enrolment goals, for safety or any other administrative reasons.

STATEMENT OF CONFIDENTIALITY

Individual participant medical information obtained as a result of this study is considered confidential and disclosure to third parties is prohibited with the exceptions noted above. Participant confidentiality will be further ensured by utilising identification code numbers to correspond to study data in the computer files.

Data generated as a result of this study will be available for inspection on request by the Sponsor, the REC and representatives of the Human Tissue Authority.

PUBLICATION POLICY

The results of genetic analysis of samples in this study shall be submitted for publication in peer reviewed journals and presentation at national and international meetings. At no

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

stage will personal identifiable information be made public or form any part of data submitted for publication or presentation.

STUDY FINANCES

Funding source

This study is funded by the Medical Research Council.

Participant stipends and payments

Participants will not be paid to participate in the study. Travel expenses will not be offered for any hospital visits in excess of usual care.

REFERENCES

1. National Institute of Clinical Excellence (NICE). CG34 Hypertension: full guideline (new recommendations and the evidence they are based on) http://guidance.nice.org.uk/CG34/Guidance/pdf/English 2006

2. World Health Organization: Global Strategy on Diet, Physical Activity and Health http://www.who.int/dietphysicalactivity/publications/facts/cvd/en/ 2009

3. NICE. Hypertension: clincial management of primary hypertension in adults. Clinical guideline CG127 http://guidance.nice.ork.uk/CG127 2011

4. Health Survey for England 2006 Latest Trends 2008

5. Dahl, L. K. Possible role of salt intake in the development of essential hypertension *Int.J.Epidemiol.* 34: 967-972, 2005

6. He FJ and Macgregor GA. Effect of modest salt reduction on blood pressure: a meta-analysis of randomized trials. Implications for public health *J Hum Hypertens.* 16: 761-770, 2002

7. Glover M, Mercier Zuber A, and O'Shaughnessy KM. Hypertension, Dietary Salt Intake, and the Role of the Thiazide-Sensitive Sodium Chloride Transporter NCCT *Cardiovascular Therapeutics* 2010

8. Gordon RD. The syndrome of hypertension and hyperkalemia with normal glomerular filtration rate: Gordon's syndrome. *Aust N Z J Med.* 16: 183-184, 1986

9. Bobulescu IA and Moe W. Luminal Na(+)/H(+) exchange in the proximal tubule *Pflugers arch.* 458: 5-21, 2009

10. Bachmann S, Bostanjoglo M, Schmitt R, and Ellison DH. Sodium-transport-related proteins in the mamalian distal nephron - distribution, ontogeny and functional aspects *Anat Embryol (Berl)* 200: 447-468, 1999

11. Simon BD, Karet FE, Rodriguez-Soriano J, Hamdan JH, DiPietro A, Trachtman H, Sanjad SA, and Lifton RP. Genetic heterogeneity of Bartter's syndrome revealed by mutation in the K^+ channel, ROMK. *Nat Genet.* 14: 152-156, 1996

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

12. Simon BD, Karet FE, Hamdan JM, DiPietro A, Sanjad SA, and Lifton RP. Bartter's syndrome, hypokalaemic alkalosis with hypercalciuria, is caused by mutations in the Na-K-Cl cotransporter NKCC2. *Nat Genet.* 13: 183-188, 1996

13. Simon DB, Nelson-Williams C, Bia MJ, Ellison DH, Karet FE, Molina AM, Vaara I, Iwata F, Cushner HM, Koolen M, and Lifton RP. Gitelman's varient of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl transporter *Nat Genet.* 12: 24-30, 1996

14. Simon BD, Bindra RS, Mansfield TA, Nelson-Williams C, Mendonca E, Stone R, Schurman S, Nayir A, Alpay H, Bakkaloglu A, Rodriguez-Soriano J, Morales JM, Sanjad SA, Taylor CM, Pilz D, Brem A, Trachtman H, Griswold W, Richard GA, John E, and Lifton RP. Mutation in the basolateral Chloride channel CLCNKB, cause Bartter syndrome type III. *Nat Genet.* 17: 171-178, 1997

15. Gitelman HJ, Graham JB, and Welt LG. A new familial disorder characterised by hypokalaemia and hypomagnesaemia *Trans Assoc Am Physicians* 79: 221-235, 1966

16. Gordon, RD, Geddes, RA, Pawsey, CGK, and O'Halloran, WO. Hypertension and severe hyperkalaemia associated with suppression of renin and aldosterone and completely reversed by dietary sodium restriction *Aust Ann Med* 4: 287-294, 1970

17. Disse-Nicodeme, S, Achard, JM, Desitter, I, Hout, A, Fournier, A, Corvol, P, and Jeunemaitre, X. A new locus on chromosome 12p 13.3 for Pseudohypoaldosteronism Type II, an autosomal dominant form of hypertension *Am.J.Hum.Genet.* 67: 302-310, 2000

18. Mansfield , TA et al. Multilocus linkage of famelial hyperkalaemia and hypertension, famelial hyperaldosteronism type II to chromosomes 1q31-42 and 17p11-21 *Nat Genet.* 16: 202-205, 1997

19. Liddle GW, Bledsoe T, and Coppage WS Jr. A familial renal disorder simulating primary aldosteronism but with negligible aldosterone secretion *Trans Assoc Am Physicians* 76: 199-213, 1963

20. Chang, SS, Grunder S, Hanukoglu A, Rösler A, Mathew PM, Hanukoglu I, Schild L, Lu Y, Shimkets RA, Nelson-Williams C, Rossier BC, and Lifton RP. Mutations in subunits of the epithelial sodium channel cause salt wasting with hyperkalaemic acidosis,Pseudohypoaldosteronism type I *Nat Genet.* 12: 248-253, 1996

21. Geller, DS, Farahi, A, Pinkerton, N, Fradley, M, Miritz, M, Spitzer, A, Meinke, G, Tsai, FT, Sigler, PB, and Lifton, RP. Activating mineralocorticoid receptor mutation in hypertension exacerbated by pregnancy. *Science* 289: 119-123, 2000

22. Sartorato P, Lapeyraque A-L, Armanini D, Kuhnle U, Khaldi Y, Solomon R, Abadie V, Di Battista E, Naselli A, Racine A, Bosio M, Caprio M, Puolet-Young V, Chabrolle J-P, Niaudet P, De Gennes C, Lecornec M-H, Poisson E, Fusco A,

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

Loli P, Lombes M, and Zennaro M-C. Different inactivating mutations of the minerolocorticoid receptor in fourteen families affected by Type I Pseudohypoaldosteronism *J Clin Endocrinol Metab.* 88: 2508-2517, 2003

23. Boyden, Lynn M., Choi, Murim, Choate, Keith A., Nelson-Williams, Carol J., Farhi, Anita, Toka, Hakan R., Tikhonova, Irina R., Bjornson, Robert, Mane, Shrikant M., Colussi, Giacomo, Lebel, Marcel, Gordon, Richard D., Semmekrot, Ben A., Poujol, Alain, Valimaki, Matti J., De Ferrari, Maria E., Sanjad, Sami A., Gutkin, Michael, Karet, Fiona E., Tucci, Joseph R., Stockigt, Jim R., Keppler-Noreuil, Kim M., Porter, Craig C., Anand, Sudhir K., Whiteford, Margo L., Davis, Ira D., Dewar, Stephanie B., Bettinelli, Alberto, Fadrowski, Jeffrey J., Belsha, Craig W., Hunley, Tracy E., Nelson, Raoul D., Trachtman, Howard, Cole, Trevor R. P., Pinsk, Maury, Bockenhauer, Detlef, Shenoy, Mohan, Vaidyanathan, Priya, Foreman, John W., Rasoulpour, Majid, Thameem, Farook, Al-Shahrouri, Hania Z., Radhakrishnan, Jai, Gharavi, Ali G., Goilav, Beatrice, and Lifton, Richard P. Mutations in kelch-like 3 and cullin 3 cause hypertension and electrolyte abnormalities *Nature* advance online publication: 2012

24. Disse-Nicodeme, S, Achard, JM, Desitter, I, Hout, A, Fournier, A, Corvol, P, and Jeunemaitre, X. A new locus on chromosome 12p 13.3 for Pseudohypoaldosteronism Type II, an autosomal dominant form of hypertension *Am.J.Hum.Genet.* 67: 302-310, 2000

25. Mansfield TA, Simon DB Farfel Z Bia M Tucci JR Lebel M Gutkin M Vialettes B Christofilis MA Kauppinen-Makelin R Mayan H Risch N Lifton RP. Multilocus linkage of familial hyperkalaemia and hypertension, pseudohypoaldosteronism type II, to chromosomes 1q31-42 and 17p11-q21. *Nat Genet.* 16: 202-205, 1997

26. Disse-Nicodeme, S, Desitter, I, Fiquet-Kempf, B, Hout, A, Stern, N, Delagousse, M, Potier, J, Ader, J, and Jeunemaitre, X. Genetic heterogeneity of famelial hyperkalaemic hypertension *J Hypertens.* 19: 1957-1964, 2001

27. Glover M, Mercier Zuber A, and O'Shaughnessy KM. Renal and Brain Isoforms of WNK3 have opposite effects on NCCT *J Am Soc Nephrol* 20: 1314-1322, 2009

28. Glover M, Mercier Zuber A, Figg N, and O'Shaughnessy KM. The activity of the thiazide-sensitive Na+–Cl–cotransporter is regulated by protein phosphatase, PP4 *Can J Physiol Pharmacol* 88: e-pub ahead of print-2010

29. Rafiqi FH, Mercier Zuber A, Glover M, Richardson C, Fleming S, Jovanovic S, Jovanovic A, O'Shaughnessy KM, and Alessi DR. Role of the WNK-activated SPAK kinase in regulating blood pressure *EMBO Mol Med* 2: 1-13, 2010

This protocol is confidential and the property of the University of Nottingham. No part of it may be transmitted, reproduced, published, or used by others persons without prior written authorisation from the University of Nottingham

9.3.5 Gordon Syndrome Patient Information Sheet

The University of Nottingham

Nottingham University Hospitals NHS

Participant Information Sheet (patients)

Final Version 1.5 14 May 2018

IRAS Project ID: 111379

Title of study: **Clinical and genetic characterisation of Gordon syndrome**

Name of Chief Investigator: Dr Mark Glover

Local researchers: Professor Ian Hall, Dr Kevin O'Shaughnessy, Wenjing Jia and Jodie Nadal

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. We'd suggest this should take about 10 minutes. Talk to others about the study if you wish. Ask us if there is anything that is not clear.

What is the purpose of the study?

The purpose of this study is to try and give us a better understanding of why some individuals develop high blood pressure (hypertension) in association with high blood potassium salt (hyperkalaemia) which is known as Gordon syndrome. Gordon syndrome is due to an inherited (genetic) tendency and although four genes have so far been identified to cause this condition there are some families where the genes have not yet been identified. In addition it is likely some patients have alterations within the four identified genes which have not yet been described.

Why have I been invited?

You have been invited to take part in this study because you have been diagnosed with Gordon syndrome. In this study we are looking for around 200 individuals who have Gordon syndrome or who are related to someone who has Gordon syndrome.

Do I have to take part?

It is up to you to decide to join the study. We will describe the study and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

What will happen to me if I take part?

If you agree to take part in the study we will obtain information about your medical problems and the medication you take from your medical records to confirm that you are eligible to take part.

If you are eligible to take part then we shall take a single blood sample (33 ml, approximately 7 teaspoons volume), a small urine sample (up to 100 ml, approximately 20 teaspoons volume, or whatever you can manage) and a collection of urine over 24 hours. The 24 hour urine collection is made into a large urine bottle and we shall provide specific instructions on how to do this.

If, as a part of your normal clinical care you subsequently begin taking a thiazide diuretic medicine to treat high blood pressure we may ask to repeat the blood and urine tests as above on a single occasion.

Expenses and payments

Participants will not be paid to participate in the study. Travel expenses will not be offered for any hospital visits in excess of usual care.

What are the possible advantages, disadvantages and risks of taking part?

We cannot promise the study will help you although your doctor will receive the results of tests performed and will discuss it with you. If a genetic alteration causing Gordon syndrome is identified in your family this would make it much easier to perform genetic testing in any other family members who may be affected in the future. Although treatment of Gordon syndrome is not currently different depending on an individual patient's genetics, if this changes in the future you may benefit from this.

The information we get from this study will help improve the treatment of high blood pressure (hypertension). Our results will be published in scientific journals however it will not be possible to identify individuals who have taken part.

There are no significant disadvantages in taking part other than the time it will take to collect the blood and urine samples as your normal treatment will be entirely unaffected by participation in this research study.

The blood test(s) may be mildly uncomfortable and may leave a small bruise. If desired we can try to arrange for the research blood test(s) to be taken at the same time as any blood tests you may need for your normal hospital care, although this may not always be possible and separate blood tests exclusively for the study may be required.

What are the possible benefits of taking part?

We cannot promise the study will help you but it is hoped that the information we get from this and future studies will improve understanding of how the kidney controls the balance of sodium salt which causes high blood pressure. We hope that this information will enable the design of new blood pressure lowering medications.

What happens when the research study stops?

Study data will be retained for at least 7 years or for longer if required. The study documents held by the Chief Investigator on behalf of the Sponsor shall be finally archived at secure

archive facilities at the University of Nottingham. This archive shall include all study databases and associated meta-data encryption codes.

Study data will be used to publish scientific and medical articles and may be used for future collaborative studies with other groups. We will ask for your consent for us to hold your contact details.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to Dr Mark Glover or another member of the research team who will do their best to answer your questions (telephone 0115 924 9924 or e-mail mark.glover@nottingham.ac.uk).

If you remain unhappy and wish to complain formally, you can do this by contacting the Patient Advice and Liaison Service (PALS), NUH NHS Trust, Freepost NEA 14614, Nottingham, NG7 1BR. Free phone 0800 183 0204 or e-mail pals@nuh.nhs.uk

In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against the University of Nottingham but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

Will my taking part in the study be kept confidential?

We will follow ethical and legal practice and all information about you will be handled in confidence.

Study data on salt levels in the blood and urine as well as genetic data will be stored in a secure computer database within the university labelled only with your initials and the participant number we will allocate to you. This means that only the research team who have access to the participant number code would be able to identify which data came from which individual participant. The participant number file is kept securely and in confidence with access limited to Professor Hall and nominated members of the research team.

The results of genetic and other tests will not be given to participants and as such your participation in the study should not affect insurance policies.

We may, if you consent (optional), also use the DNA (genetic material) from your blood samples for further genetic sequencing (analysis) with new genetic techniques as they become available, either within the University of Nottingham or with collaborators at other institutions. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.

If you join the study, we will use information collected from you your medical records during the course of the research. This information will be kept **strictly confidential**, stored in a secure and locked office, and on a password protected database at the University of Nottingham. Under UK Data Protection laws the University is the Data Controller (legally

responsible for the data security) and the Chief Investigator of this study (Professor Ian Hall) is the Data Custodian (manages access to the data). This means we are responsible for looking after your information and using it properly. Your rights to access, change or move your information are limited as we need to manage your information in specific ways to comply with certain laws and for the research to be reliable and accurate. To safeguard your rights we will use the minimum personally – identifiable information possible.

You can find out more about how we use your information and to read our privacy notice at:

https://www.nottingham.ac.uk/utilities/privacy.aspx.

The data collected for the study will be looked at and stored by authorised persons from the University of Nottingham who are organising the research. They may also be looked at by authorised people from regulatory organisations to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty.

Your contact information will be kept by the University of Nottingham for 7 years after the end of the study so that we are able to contact you about the findings of the study and possible follow-up studies (unless you advise us that you do not wish to be contacted). This information will be kept separately from the research data collected and only those who need to will have access to it. All other data (research data) will be kept securely for 7 years. After this time your data will be disposed of securely. During this time all precautions will be taken by all those involved to maintain your confidentiality, only members of the research team given permission by the data custodian will have access to your personal data.

In accordance with the University of Nottingham's, the Government's and our funders' policies we may share our research data with researchers in other Universities and organisations, including those in other countries, for research in health and social care. Sharing research data is important to allow peer scrutiny, re-use (and therefore avoiding duplication of research) and to understand the bigger picture in particular areas of research. Data sharing in this way is usually anonymised (so that you could not be identified) but if we need to share identifiable information we will seek your consent for this and ensure it is secure. You will be made aware then if the data is to be shared with countries whose data protection laws differ to those of the UK and how we will protect your confidentiality.

What will happen if I don't want to carry on with the study?

Your participation is voluntary and you are free to withdraw at any time in the first two weeks following your entry into the study, without giving any reason, and without your legal rights being affected. If you withdraw then the information collected so far cannot be erased and that this information may still be used in the project analysis. Any remaining samples will be destroyed if you wish. If you wish to withdraw from the study you should ask to speak to Dr Mark Glover (telephone 0115 924 9924 or e-mail mark.glover@nottingham.ac.uk)

Involvement of my hospital doctor and General Practitioner / Family doctor (GP)

If you choose to participate the doctor(s) looking after you in hospital and your GP will be informed of your involvement in this study and a copy of your consent form filed in your medical records.

What will happen to any samples I give?

The blood sample(s) will be analysed for blood salt levels and DNA in your blood will be analysed to look for variations in the genes that control salt and water balance. The urine samples will be analysed for salt and protein levels.

We would also like to seek your consent so that any remaining samples may be stored and used in possible future research – this is optional (please indicate you agree to this on the consent form). The samples will be stored with a code unique to you and securely at the University of Nottingham under the University's Human Tissue Research Licence (no 12265).

Some of these future studies may be carried out by researchers other than current team of Drs Glover and O'Shaughnessy, who run this study, including researchers working for commercial companies. Any samples or data used will be anonymised, and you will not be identified in anyway. If you do not agree to this any remaining samples will be disposed of in accordance with the Human Tissue Authority's codes of practice.

In addition, if you agree, we may contact you directly in the future about further Gordon syndrome related research projects although you have no obligation to take part in either this or any future study.

Will any genetic tests be done?

Yes. DNA in your blood will be analysed to look for variations in the genes that control salt and water balance.

What will happen to the results of the research study?

Our results will be published in scientific journals however it will not be possible to identify individuals who have taken part. The detailed nature of this research and the tests involved means that it may be a year or more before results are published. We are not currently in a position to identify exactly in which scientific journal this will be.

A summary of the study results will be on our departmental website when they are available. The website address is:

http://www.nottingham.ac.uk/medicine/about/respiratorymedicine/index.aspx

Who is organising and funding the research?

This research is organised by clinical academic researchers in the Division of Respiratory Medicine at the University of Nottingham and the Clinical Pharmacology Unit at the University of Cambridge. Funding is from the Medical Research Council.

No member of the research team will receive payment over and above their usual employment as clinicians and academics as a result of your participation in this study.

Who has reviewed this study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by the NRES Committee East Midlands- Northampton.

Further information and contact details

Further information is available from Dr Mark Glover (chief investigator), Division of Respiratory Medicine, D Floor, South Block, Queen's Medical Centre, Nottingham, NG7 2UH. E-mail: mark.glover@nottingham.ac.uk, telephone 0115 924 9924

We would like to thank you for taking the time to read this document. Please ask us if you have any questions.

9.3.6 Gordon Syndrome Regulatory Sheet

REISSUED: 30.11.2012 Due to error or version and dates within document listing

NHS
Health Research Authority

NRES Committee East Midlands - Northampton
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Telephone: 0115 8839435
Facsimile: 0115 8839294

31 August 2012

Dr Mark Glover
Clinical Lecturer and Honorary Specialist Registrar
University of Nottingham and Nottingham University Hospital NHS Trust
Division of Therapeutics and Molecular Medicine
University of Nottingham, QMC, D Floor South Block
NG7 2UH

Dear Dr Glover

Study title:	**Clinical and genetic characterisation of Gordon's syndrome**
REC reference:	**12/EM/0317**
Protocol number:	**12082**

Thank you for your letter of 23 August 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

REISSUED: 30.11.2012 Due to error or version and dates within document listing

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Evidence of insurance or indemnity		25 July 2012
GP/Consultant Information Sheets	1.0	24 July 2012
Investigator CV		
Letter from Sponsor		27 July 2012
Participant Consent Form	**1.1**	**23 August 2012**
Participant Information Sheet: Patients	1.1	23 August 2012
Participant Information Sheet: Family Members	1.1	23 August 2012
Protocol	1.1	23 August 2012
REC application	111379/3478 54/1/420	26 July 2012
Response to Request for Further Information		23 August 2012

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion,

REISSUED: 30.11.2012 Due to error or version and dates within document listing

including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/EM/0317	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely



Mr Paul Hamilton
Chair

Email: georgia.copeland@nottspct.nhs.uk

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Mr Paul Cartledge*

Dr Maria Koufali, Research & Development, Nottingham University Hospitals NHS Trust