Dynamics of Iodine Fluctuations: Effects on Dietary Intake in the UK Population, In Vitro Models, and Cellular Stress Response

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Abstract

Iodine is essential for the human body as it is crucial for the synthesis of the thyroid hormones thyroxine (T4) and triiodothyronine (T3) by the thyroid gland. The World Health Organization (WHO) recognised that iodine deficiency is a significant risk factor that affects growth and development from early life, as well as throughout all ages.

Iodine-rich food sources, including fish, milk, dairy products, and eggs, of which milk and dairy products are the predominant source of iodine intake in many industrialised countries, including the UK.

Incorporating iodine fortification into salt has proven to be an effective public health intervention for preventing iodine deficiency disorders in populations with insufficient iodine intake. Moreover, iodine intake poses a significant nutritional challenge in modern diets, especially for specific population groups, such as vegetarians and vegans. Consuming iodine-rich foods suitable for vegans, such as seaweed, might only provide a significant amount intermittently, causing fluctuations in their iodine intake levels. This raises the question of health outcomes related to iodine fluctuations. The lack of understanding of both public health and in vitro experiments is what we have keen on research. In this study, we attempted to analyse this situation from the perspective of public health and in vitro experiments.

First, from the most recent NDNS program, the national population iodine status in the UK was studied. The results indicated that an adequate iodine level was assessed from the median UIC in the UK population, with a borderline to deficiency level of median UIC in reproductive-age females(100ug/L).The primary sources of iodine intake for all age and sex groups were milk and dairy products, accounting for 34.28% and 52.19% of the total iodine intake, respectively. In addition, we used the standard deviation (SD) of the four-day food diary that recorded iodine intake as a fluctuation indicator, analysed the relationship between the fluctuation to UIC (μg/L) and mean iodine intake(μg)

from the population, and the results showed a strong correlation between the fluctuation indicator and mean iodine intake, and a potential weak correlation between UIC and the fluctuation indicator. Furthermore, fish and fish products were the main contributors to fluctuations in iodine intake.

Next, an in vitro iodine fluctuation model was built to analyse the effects of fluctuating iodine on thyroid hormone synthesis pathways. With the successfully built model, using the human thyroid cell line Nthy-roi 3-1 we observed the interaction between TSH and the fluctuation of iodine levels affecting thyroid hormone production-related genes, including NIS, TSHR, PAX8, DUOX2 PDS, and TPO. Additionally, the ER stress indicator GRP78 was significantly increased by TSH stimulation. The autophagy indicator shows an upregulation during the iodine starvation phase of the fluctuation model, which indicates that the thyroid cells used autophagy to maintain homeostasis.

Furthermore, the T3 effect indicated that it could significantly increase the TH target cell proliferation rate, including HepG2, C2C12, and HK2. However, only HepG2 cells showed a significant effect from T3 which aids its recovery from H2O2-induced oxidative stress.

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Table of Contents

Table of Contents

Abstract i

Acknowledgements iii

Table of Contents iv

List of Figures ix

List of Tables xi

List of Abbreviations xiii

Chapter 1 General Introduction 1

1.1 Overview 2

1.2 The importance of iodine 3

1.2.1 Iodine as an environmentally occurring nutrient. 3

1.2.2 Role of iodine in thyroid function and cellular metabolism 4

1.2.3 Historical perspective on iodine 6

1.2.4 Recommended iodine intake 6

1.2.5 Dietary sources of iodine 7

1.2.6 Assessment of iodine status 11

1.2.7 Policy of iodine fortification and global iodine status in recent years 12

1.2.8 Iodine status in UK 16

1.2.9 Iodine status with Vegan/Vegetarian 17

1.2.10 Trends and patterns of iodine intake fluctuation 19

1.3 Integrated Perspectives on Thyroid Physiology 21

1.3.1 General information of Thyroid 21

1.3.2 Biosynthesis of Thyroid Hormones 22

1.3.3 Thyroid Hormone Transportation 23

1.3.4 Thyroid Hormone Action and Mechanisms 29

1.3.5 Regulation of Thyroid Function 32

1.3.6 Oxidative Stress and Thyroid Function 34

1.3.7 Pathophysiology of Thyroid Disorders 36

1.4 Aim and Objectives 38

Chapter 2 General Method 40
2.1 Cell culture 41
2.1.1Used Cell lines 41
2.1.2 For splitting cells 44
2.1.3 Culturing cells 44
2.1.4 Freezing and thawing cells 44
2.1.5 Cell counting 45
2.2 Cell viability 46
2.2.1 MTT 46
2.2.2 CCK8 ASSAY 47
2.3 Reactive Oxygen Species (ROS) Measurement 49
2.4 Enzyme-linked immunosorbent assay (ELISA) 50
2.4.1 Plate Preparation 50
2.4.2 Assay Procedure 50
2.5 Real-Time Quantitative PCR 51
2.5.1 Sample collection for RNA extraction 51
2.5.2 RNA extraction 51
2.5.3 Quantify total RNA using Nanodrop 52
2.5.4 cDNA synthesis 53
2.5.5 Primer Design 53
2.5.6 Real time quantitative PCR (RT PCR) 55
2.5.7 Quantification of the transcript abundance using standard curve 57
2.6 Western Blot 58
2.6.1 Preparation of protein reagents 58
2.6.2 Western blot sample collection 59
2.6.3 Quantification of protein concentration using 2D Quant 59
2.6.4 Polyacrylamide Gel Electrophoresis (PAGE) 60
2.6.5 Incubate with primary and secondary antibodies and Detection 63
2.7 Statistics 65
Chapter 3 The assessment of iodine intake of UK population using the National Diet and Nutrition Survey Rolling Programme (NDNS RP). 66
3.1 Introduction 67

3.2 Method 74
3.2.1 The National Diet and Nutrition Survey Rolling Programme (NDNS RP) and the survey population. 74
3.2.2 Iodine level analysed by the urine iodine concentration (UIC) sample. 75
3.2.3 Assessing fluctuation levels using standard deviation (SD) 77
3.2.4 Contribution of different foods to iodine intake 78
3.2.5 Data Analysis 78
3.3 Results 80
3.3.1 The observation of current UK iodine status using UIC from NDNS ... 80
3.3.2 Overall dietary iodine level fluctuation rating and its association with iodine status 81
3.3.3 Regional comparison iodine status dietary fluctuation 83
3.3.4 Assessment of the association between intake variation and absolute intake levels. 85
3.3.5 The composition percentage of different iodine-rich foods in the NDNS four-day food diary in different age groups 91
3.3.6 Seaweed consumption among NDNS respondents. 106
3.4 Discussion 108
Iodine status from NDNS 108
The fluctuation score and its relationship with UIC 109
The approach of spot urine sample used in NDNS 112
Iodine-rich Food contributions 113
Limitations 115
Chapter 4 Exploring the Impact of Iodine Fluctuations: In Vitro Studies on Thyroid Hormone Production and Cellular Stress Response 117
4.1 Introduction 118
Iodine Transport, Thyroid Hormone Biosynthesis, and Regulatory Mechanisms 118
Assessment of the impact of iodine fluctuation on thyroid cells in vitro. .. 123
4.2 Method 125
4.2.1 Cell culture 125
4.2.2 Composition of the solution and treatment condition 125

4.2.3 Determination of iodine tolerance in thyroid cell 126
4.2.4 The application of the Transwell model to form an in vitro thyroid follicle unit. 126
4.2.5 The design of the Fluctuation model..... 128
4.2.6 fluctuation sample collection 130
4.2.7 Assessment of TSH-stimulated cellular ROS rate and cell sensitivity to exogenous ROS in the Nthy-ori 3-1 cell line. 130
4.2.8 Statistical Analyses..... 132
4.3 Result 133
4.3.1 The determination of iodine tolerance level in thyroid cells 133
4.3.2 Impact of Iodine Fluctuations on TSH-Regulated Gene Expression in Thyroid Signalling Pathways 135
4.3.3 Effects of Iodine Deprivation and Reintroduction on NIS mRNA and Protein Expression Mediated by TSH Interaction 137
4.3.4 Effects of TSH and Iodine Fluctuations on the Expression of Iodide Transport and Oxidation Genes in Thyrocytes 140
4.3.5 Effects of Iodine Deprivation and Reintroduction on TGF-β1 mRNA Expression in Response to TSH and Iodine Concentrations 142
4.3.6 Impact of Iodine Fluctuation on ER Stress and Autophagy Markers GRP78 and LC3B under TSH Stimulation..... 143
4.3.7 The Fluctuation of iodine does not shown interaction on affecting ROS level in the current model design..... 148
4.3.8 The continuous of iodine incubation shown interaction on affecting ROS level in the thyroid cell model 153
4.3.9 Assessment of the transwell model system to explore thyroid cell responsiveness to iodine fluctuations. 155
4.4 Chapter Discussion..... 156
Chapter 5 The effect of Thyroid hormone (T3) on target cell lines 166
5.1 Introduction..... 167
5.1.1 Function of thyroid hormone..... 167
5.1.2 Thyroid hormone and oxidative stress..... 169
5.1.3 Oxidative Stress Association of Hypothyroidism..... 170
5.1.4 Bridging iodine fluctuations with cellular responses to THs..... 172
5.1.5 Aim and hypothesis 173

5.2 Method 174
5.2.1 Cell culture 174
5.2.2 Composition of the treatment media 174
5.2.3 Using the cell counting equipment to analyze the effect of T3 treatment on cellular proliferation rate in different cells 175
5.2.4 Investigation of the effect of T3 towards cellular stress via Cell viability assay 176
5.2.5 Statistical Analysis 178
5.3 Result 179
5.3.1 Thyroid hormones (T3) are capable of inducing proliferation in HK2, HepG2 and C2C12 cells. 179
5.3.2 No protective effect of T3 treatment was observed with tested cell lines after 24h of T3 treatment. 181
5.3.3 Compare the difference of HFM and SFM media 183
5.3.4 TH have protection effect against H_2O_2 on target cell line HepG2 but no other tested cell lines 184
5.3.5 Effect of T3 treatment on target cell line HEK293 under different concentration of H2O2 induced oxidative stress 190
5.4 Discussion 193
Chapter 6 Discussion, Limitation and Conclusion 199
6.1 Analysing Iodine Status and Fluctuations in the UK: Insights from the UIC and Primary Dietary Sources. 201
6.1.1 Limitation of using the NDNS as a population database 201
6.2 Building the fluctuation in vitro model. 202
6.2.1 Limitations 203
6.3 Thyroid hormone (T3) was able to up-regulate the proliferationrate but no protective effect with target cell lines. 204
6.4 Conclusion 205
6.5 Future works 205
Chapter 7 References 207

List of Figures

Figure1. 1 Cycle of iodine.....4
Figure1. 2 Structure of thyroid hormones and related precursor components .5
Figure1. 3 **Iodine concentrations of seafood samples** 10
Figure1. 4 **Percentage of households consuming iodized salt.** 15
Figure1. 5 **Global iodine status in 2021**.....16
Figure1. 6 **The location and structure of human thyroid gland**.....21
Figure1. 7 **Thyroid hormone synthesis, secretion, and major signaling pathways in thyrocytes**.....24
Figure1. 8 **Thyroid hormone and deiodinase.** 29
Figure1. 9 **General model for thyroid hormone (T3) action in the nucleus.** 31
Figure1. 10 **The hypothalamic–pituitary–thyroid axis.**.....33

Figure 2. 1 MTT converted to formazan.....48
Figure 2. 2 WST-8 Assay Mechanism.....48

Figure 3. 1 **Correlation Between Urinary Iodine Concentration (UIC) and Standard Deviation (SD) of Daily Iodine Intake in NDNS Participants** 88
Figure 3. 2 **Correlation Between Urinary Iodine Concentration (UIC) and Mean Daily Iodine Intake(μg) in NDNS Participants**.....89
Figure 3. 3 **Correlation Between Mean Daily Iodine Intake(μg) and Standard Deviation (SD) of Daily Iodine Intake in NDNS Participants** 90

Figure 4. 1 Reproduced from the Werner & Ingbar's The Thyroid: A Fundamental & Clinical Text, 10th Edition Section1.Chapter 4B. Thyroid Hormone Synthesis. cAMP, cyclic AMP; DIT, diiodotyrosine; DUOX, dual oxidase 2; MIT, monoiodotyrosine; NIS, sodium/iodide symporter; PDS, pendrin; PKA, protein kinase A; TG, thyroglobulin; TPO, thyroid peroxidase; TSH, thyrotropin; TSHR, TSH receptor. The graph was generated with Biorender.com 120
Figure 4. 2 **Transwell protocol** 127
Figure 4. 3 **Transwell model and 6 well model as comparison indication**127
Figure 4. 4 **Timeline of treatment groups for fluctuation model.** 129
Figure 4. 5 **Timeline of treatment groups for continuous iodine incubation model.**..... 132
Figure 4. 6 **Cell viability of Nthy-ori 3-1 with a range of potassium iodide (KI) to measure the iodide tolarence level with the cell**..... 134

Figure 4. 7 TSHR and PAX8 expression in different phase of the fluctuation model. 136
Figure 4. 8 **Sodium/Iodide symporter (NIS) protein and mRNA expression in the two phases of the fluctuation cycle** 139
Figure 4. 9 **mRNA expression of genes related to thyroid hormone production pathway in the two phases of the fluctuation model.** 141
Figure 4. 10 **TGF-** β1 **mRNA expression in the two phases of the fluctuation cycle** 142
Figure 4. 11 **GRP78 protein expression in the two phases of the fluctuation cycle** 144
Figure 4. 12 **LC3BI&II protein expression in the two phases of the fluctuation cycle** 147
Figure 4. 13 **ROS level monitored at cycle three down and up timepoint of the Fluctuation model on thyroid cells** 150
Figure 4. 14 **Effect of iodide fluctuation at the down point with Nthy-ori 3-1 cell** 151
Figure 4. 15 **Effect of iodide fluctuation at the up point with Nthy-ori 3-1 cell** 152
Figure 4. 16 **ROS level with continuous incubation of TSH(+/-) and iodide (+/-) on thyroid cells** 154
Figure 4. 17**standard curve of the ELISA assay** 155

Figure 5. 1 **Thyroid hormone transport, metabolism and action in a target cell. Reproduced from Schwartz and Stevenson (2007)** 169
Figure 5. 2 Building the Hormone-free cellular model. 176
Figure 5. 3 Protocol of the oxidative stress with different treatment 177
Figure 5. 4 **Effect of 24h T3 treatment on different cell lines** 180
Figure 5. 5 **Effect of H2O2 induced cellular stress** 182
Figure 5. 6 **Cell viability comparison with target cell line.** 183
Figure 5. 7 **The effect of oxidative stress followed with 24h or 48h Hormone-free medium (HFM) +/-T3 treatment in C2C12 cells.** 186
Figure 5. 8 The effect of oxidative stress followed with 24h or 48h Serum-free medium (SFM) +/-T3 treatment in HK2 cells. 188
Figure 5. 9 **The effect of oxidative stress followed with 24h or 48h Hormone-free medium (HFM) +/-T3 treatment in HepG2 cells.** 190
Figure 5. 10 The effect of oxidative stress followed with 24h or 48h Hormone-free medium (HFM) +/-T3 treatment in HEK293 cells. 192

List of Tables

Table 1. 1Recommendations for iodine intake (µg/d) by age group 7
Table 1. 2 **Estimated iodine content of foods in UK** 9
Table 1. 3**The standard for assessment for iodine nutrition status in different population groups on UIC**..... 13
Table 1. 4 **Main characteristics of the major thyroid hormone transporters in humans.** 26
Table 1. 5 **Human thyroid hormone transporter** 28
Table 1. 6 **Characteristics of Deiodinases** 28

Table 2. 1 **Seeding density of different plate types in cell culture**..... 46
Table 2. 2 Step 1 of RevertAid RT Kit cDNA synthesis mixture reagents 54
Table 2. 3 Step 2 of RevertAid RT Kit cDNA synthesis mixture reagents 54
Table 2. 4 RT PCR primers for human gene measured 55
Table 2. 5 Volumes of the reaction reagent used in RTqPCR in each reaction well. 56
Table 2. 6 RT qPCR SYBR Green program template used for Roche LightCycler 480. 57
Table 2. 7 The components in 10mL 2X SDS 58
Table 2. 8 Quantity of components used to make transfer buffer. 62
Table 2. 9 Quantity of components for 10x TBS 63
Table 2. 10 Quantity of components for Stripping Buffer..... 65

Table 3. 1 **The age cohort and actual participants used in the NDNS year9-11 (2020)** 75
Table 3. 2 **Urinary iodine Concentration (UIC) cut-off values for public health significance indicator and specified rating**..... 76
Table 3. 3 **Distribution of Iodine Intake Variability Measured by Standard Deviation (SD) Across Percentiles** 77
Table 3. 4 **Fluctuation score for indicating iodine intake irregularity** 78
Table 3. 5 **UIC status in UK population from NDNS Year 9-11.** 81
Table 3. 6 **UIC status in different fluctuation rating groups from NDNS Year 9-11.**..... 82
Table 3. 7 **UIC status in different countries from NDNS yr 9-11** 84
Table 3. 8 **Fluctuation rating proportion in different countries from NDNS yr 9-11.**..... 84
Table 3. 9 **Percentage contribution of food and food groups to daily iodine intake for male participants aged 4 to12 years. NDNS years 9-11**..... 93

Table 3. 10 **Percentage contribution of food and food groups to daily iodine intake for female participants aged 4 to12 years. NDNS years 9-11** 94
Table 3. 11 **Percentage contribution of food and food groups to daily iodine intake for male participants aged 13 to18 years. NDNS years 9-11** 97
Table 3. 12 **Percentage contribution of food and food groups to daily iodine intake for female participants aged 13 to18 years. NDNS years 9-11** 98
Table 3. 13 **Percentage contribution of food and food groups to daily iodine intake for male participants aged 19 to 60 years. NDNS years 9-11** 100
Table 3. 14 **Percentage contribution of food and food groups to daily iodine intake for female participants aged 19 to60 years. NDNS years 9-11** 101
Table 3. 15 **Percentage contribution of food and food groups to daily iodine intake for male participants aged over 60 years. NDNS years 9-11** 104
Table 3. 16 Percentage contribution of food and food groups to daily iodine intake for female participants aged over 60 years. NDNS years 9-11 105
Table 3. 17 **Frequency distribution of seaweed consumer separated by gender, age groups and survey years.** 107

Table 4. 1 Solution and treatment condition of fluctuation model 129
Table 4. 2 **Result OD value reading and fT4 concentrations in transwell model using ELISA** 156

List of Abbreviations

AKI: acute kidney injury

ANOVA: Analysis of variance

BDA British Dietetic Association

BSA: Bovine Serum Albumin

cAMP: cyclic adenosine monophosphate

CCK: Cell Counting Kit

cDNA: Complementary DNA

CI: confidence interval

CKD: chronic kidney disease

DAG: diacylglycerol

DIT: diiodotyrosine

DMEM: Eagle's minimal essential medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic Acid

DTT: DL-Dithiothreitol

DUOX1: Dual oxidases 1

DUOX2: Dual oxidases 2

EDC: endocrine-disrupting chemical

ELISA: enzyme-linked immunosorbent assay

ER: endoplasmic reticulum

ESFA European Food Safety Authority

FBS: Foetal Bovine Serum

FFQs food frequency questionnaires

FOXE1 forkhead box protein E1

GADPH: glyceraldehyde-3-phosphate dehydrogenase

GI: gastrointestinal symptoms

GPCR: G-protein-coupled receptor

GRP78: glucose-regulated protein 78kda

H2O2: Hydrogen peroxide

HHEX hematopoietically-expressed homeobox protein

HPLC high-performance liquid chromatography

HT: Hashimoto thyroiditis

ICP-MS inductively coupled plasma mass spectrometry

IDD: iodine deficiency disorders

IGN: Iodine global network

IP3: inositol triphosphate

IQ intelligence quotient

IQR interquartile range

KI: Potassium Iodide

LC3B: Light chain 3 B

LRNI lower reference nutrient intake

L-T4: Levo-Thyroxine 4

MAFF ministry of Agriculture Fisheries and Food

MCT10: monocarboxylate transporter 10

MCT8: monocarboxylate transporter 8

MDA: malondialdehyde

MIC: milk iodine concentration ()

MIT: monoiodotyrosine

MPO: myeloperoxidase

mRNA: Messenger RNA

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NAFLD: non-alcoholic fatty liver disease

NaI: Sodium Iodide

NDB: UK Nutrient Databank

NDNS: National Diet and Nutrition Survey

NIS: Sodium Iodide Symporter

NKX2-1 NK2 homeobox 1

NOX: NADPH oxidase

Nox4: NADPH oxidase 4

OATP1C1: organic anion transporting polypeptide 1C1

PAGE: Polyacrylamide Gel Electrophoresis

PAX8: Paired box gene 8

PBS: Phosphate Buffer Saline

PDS: pendrin

Pen-Strep: Penicillin/Streptomycin

PTU: propylthiouracil

RNI Reference nutrient intake

ROS: Reactive oxygen species

RPMI: Gibco Roswell Park Memorial Institute

RT qPCR: Real Time Quantitative Polymerase Chain Reaction

SAC: school-aged children

SD: Standard Deviation

SDS: Sodium Dodecyl Sulfate

SEM: Standard error of means

SOD: superoxide dismutase

T3: 3,3′, 5-Triiodothyronine

T4: Thyroxine

TAC: total antioxidant capacity

TBGs: thyroxine-binding globulins

TG: thyroglobulin

TGF-b1: Transforming growth factor beta 1

TH: Thyroid Hormones

TPO: thyroid peroxidase

TSH: Thyroid Stimulating Hormone

TSHR: Thyroid Stimulating Hormone Receptor

TTFs thyroid transcription factors

UIC: Urinary Iodine Concentration

UNICEF United Nations Children's Fund

UPR: unfolded protein response

USI: universal salt iodization

UV: Ultraviolet

VIP: vasoactive intestinal peptide

WHO: World Health Organization

Chapter 1 General Introduction

1.1 Overview

Iodine is essential for normal human development, growth, and metabolism, as it is necessary for the synthesis of the thyroid hormones thyroxine (T4) and triiodothyronine (T3) by the thyroid gland(Kopp and Solis-S, 2009). Thyroid hormones play a pivotal role in regulating essential metabolic functions necessary for the proper growth and development of infants, as well as in maintaining metabolic processes in adults (Brent, 2012, Cheng et al., 2010). Aside from its role in thyroid hormone synthesis, research indicates that iodine, primarily in the form of iodide, possesses antioxidant properties by removing free radicals and safeguarding cells against oxidative stress(Küpper et al., 2008, Dijck-Brouwer et al., 2022).

Several food sources are naturally rich in iodine, including fish, dairy products, and eggs, which are considered the primary sources of dietary iodine(Dahl et al., 2004, Nerhus et al., 2018). Additionally, seaweed has gained attention for its high iodine content outside of where it is traditionally consumed, such as in Asian cuisine(Stig Andersen, 2019). Owing to the increasing attention paid to iodine deficiency, incorporating iodine fortification of salt has proven to be an effective public health intervention in preventing iodine deficiency disorders in populations with insufficient iodine intake(F. DELANGE, 1999, Sebotsa et al., 2005, Morales-Suárez-Varela et al., 2018). Furthermore, iodine intake poses a significant nutritional challenge in modern diets, especially for specific population groups, such as vegetarians and vegans, who may not consume traditional iodine-rich foods. Studies have indicated that vegans and vegetarians tend to have lower median urinary iodine concentrations than omnivores, with vegans being the lowest, suggesting a suboptimal iodine status. Individuals following diets that exclude iodine-rich foods have been found to have inadequate iodine intakes (Eveleigh et al., 2020, Eveleigh et al., 2023). Additionally, when consuming iodine-rich foods suitable for vegans, such as seaweed, they might only receive a significant amount intermittently, causing a fluctuation in their iodine intake levels. Similarly, the general population is not immune to fluctuating iodine intakes. As no existing study focused on the fluctuations of iodine intake and their subsequent impact on

thyroid health. This gap limits our understanding of how varying iodine levels influence thyroid function and related health outcomes, both at the population and biological levels. Investigating these dynamics through both public health studies and experimental approaches, such as in vitro cellular models or animal models, would provide critical insights into the physiological mechanisms at play.

1.2 The importance of iodine

1.2.1 Iodine as an environmentally occurring nutrient.

Iodine is considered to be an essential micronutrient (Gropper et al., 2013). Iodine is naturally present in the earth's environment as iodide salts, but its distribution is not consistent. The quantity of iodine found is greatly affected by soil type and location, with soils generally having higher concentrations of iodine than rocks. Regions where people depend heavily on crops grown in areas with lower levels of iodine in the soil are susceptible to deficiencies (Bender and Cunningham, 2021).

The predominant source of iodine in the soil is atmospheric iodine, which is transported to the soil by rainwater. This rainwater is derived from the oceans, where the global iodine cycle is primarily from. Seawater contains an average of 58 μg/L iodine, which is significantly higher than the lower and variable levels found in non-saline surface waters (Fuge and Johnson, 1986).

Iodine in seawater is present predominantly as iodide (I^-) and iodate (IO_3^-), which accumulate in microalgae, such as phytoplankton and coastal macrophytic algae. Ionization of iodine is the primary method through which it enters the atmosphere. This process changes ionic iodine into its molecular form (I_2), which is then transported into the lower part of the atmosphere near the ocean's surface. Eventually, the iodine is brought back to the ground by rainfall (Figure 1.1) (Leblanc et al., 2006, Chance et al., 2009).

Dietary iodine can be found in foods as either organic iodine, which is bound to amino acids, or as iodides or iodates. Since ionic iodine is more readily absorbed by the body, organic iodine must first be freed and converted, whereas iodate must be reduced within the gastrointestinal tract (Gropper et

al., 2013). Iodide is quickly absorbed from the stomach and, to a lesser extent, from the duodenum, where it travels through the bloodstream to the thyroid gland, which contains approximately 70%–80% of the body's iodide(Gropper et al., 2013).

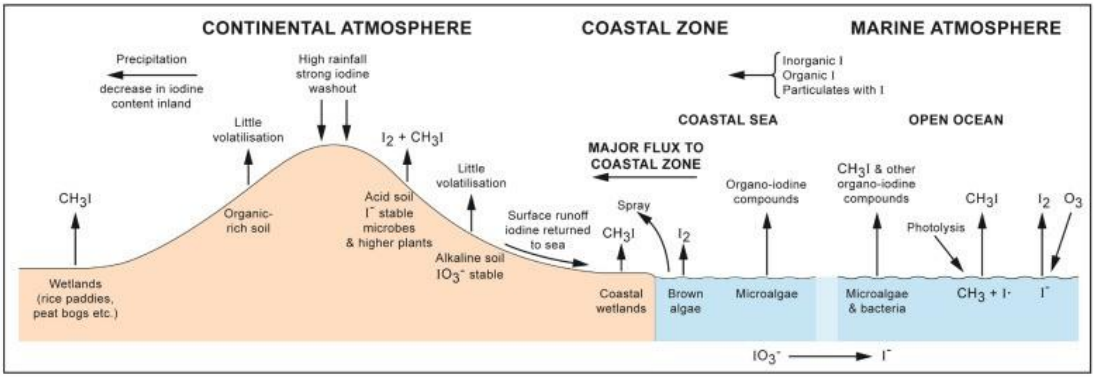


Figure1. 1 Cycle of iodine
The illustration of global geochemical cycle of iodine. Figure is taken from Fuge and Johnson (2015) Iodine and human health, the role of environmental geochemistry and diet, a review, Applied Geochemistry, Pages 282-302.

1.2.2 Role of iodine in thyroid function and cellular metabolism

The production of thyroid hormones (triiodothyronine (T3) and thyroxine (T4)) relies on the presence of iodine, which is a vital nutrient obtained from dietary sources. The majority of iodine, approximately 70-80%, is stored in the thyroid gland of healthy adults(Ahad and Ganie, 2010). The primary function of the thyroid gland is to produce thyroid hormones, which are crucial for their synthesis and secretion. There are two major forms of thyroid hormones (THs), thyroxine (T4) and 3,3′, 5-Triiodothyronine(T3), and their structures are shown in Figure 1.2. T4 is considered a prohormone that undergoes deiodination to form T3, the active form of the thyroid hormone. T4 has limited biological activity in contrast to T3. THs play an essential role in regulating metabolic processes that are necessary for early life growth and development, as well as in the regulation of metabolic activities in adults (Brent, 2012, Cheng et al., 2010). These hormones can affect tissue growth, blood pressure, metabolic rates, and glucose oxidation. THs regulate metabolism via the brain, white and brown fat tissues, skeletal muscle, liver, and

pancreas(Boelaert and Franklyn, 2005). In addition, by regulating energy storage and consumption, TH affects important metabolic processes linked to energy balance (Cheng et al., 2010, Liu and Brent, 2010, Iwen et al., 2013).

The process of secretion of T3 and T4 is composed of several complicated steps, which are summarised below. Further details are discussed in a subsequent section. The initial step involves the activity of thyroid peroxidase (TPO), which facilitates iodination of tyrosyl residues in thyroglobulin (TG), also known as organification. This leads to the formation of monoiodotyrosine (MIT) and diiodotyrosine (DIT). Subsequently, two iodotyrosines are joined together through a coupling reaction catalysed by TPO, resulting in the production of either T4 or T3 (Kopp and Solis-S, 2009).

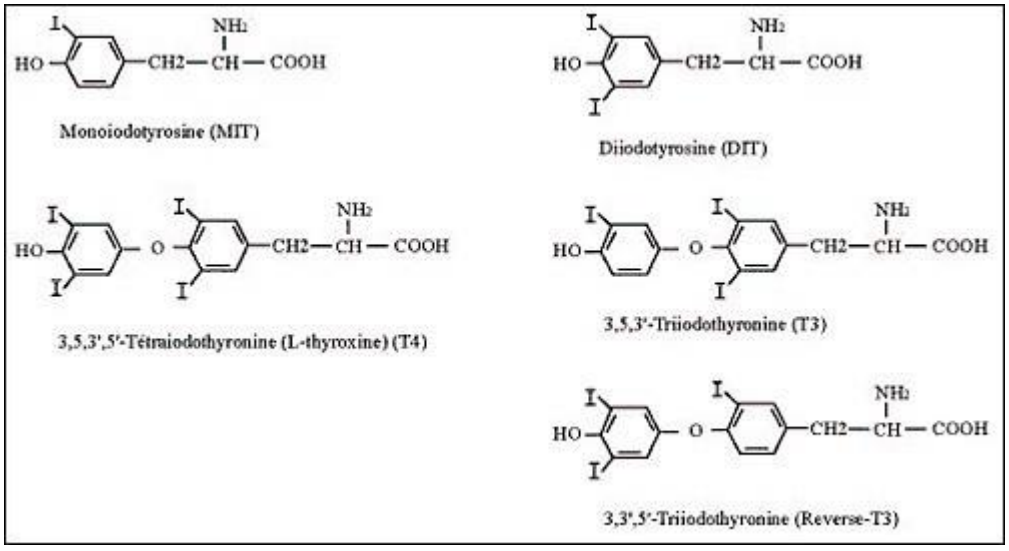


Figure1. 2 Structure of thyroid hormones and related precursor components
Overview of the structure of thyroid hormones (T3 and T4) and related precursor components. Figure is taken from Rousset B, Dupuy C, Miot F, et al. (2015) Chapter 2 Thyroid Hormone Synthesis and Secretion. www.THYROIDMANAGER.ORG

1.2.3 Historical perspective on iodine

By the early 1600s, anatomists had recognised the thyroid gland in humans. However, diseases related to the thyroid gland were not recognised until the 1820s as iodine was used as a treatment for patient with goitres (Braverman and Cooper, 2013). The World Health Organization (WHO) published the first global estimate of the prevalence of goitre in 1980, estimating that 20–60% of the world's population was iodine deficient and/or goitrous, with developing countries facing most of the burden (Zimmermann, 2009).

Iodine deficiency has a number of detrimental impacts on human and animal particularly growth and development systems. These conditions are collectively known as "iodine deficiency disorders syndromes" (IDDs). People who suffer from low iodine consumption lead to insufficient thyroid hormone production and this can cause various health consequences on individuals at all ages.

Pregnant women typically have low iodine storage in areas with long-term iodine deficiency. Pathological alterations, such as goitre and hypothyroidism, may develop if insufficient thyroidal iodine is available to meet the increased maternal iodine requirement. IDDs could also affect brain development. Indeed, people living in areas with chronic iodine deficiency conditions may suffer from impaired intelligence quotient (IQ), leading to reduced mental function and slowed physical growth, especially for individuals in their childhood or adolescence (Hetzel, 1983, Zimmermann et al., 2008, Zimmermann, 2009).

1.2.4 Recommended iodine intake

The iodine intake recommendations between the UK and WHO are shown in Table 1.1. The daily recommendation of iodine intake from the WHO (2007) is as follows: in children aged 0–5 years, 90 μg; in children aged 6–12 years, 120 μg; in adults, 150 μg; and in pregnancy and lactation, 250 μg. This is different from the UK recommendations,140μg/day compared to 150μg/day from the WHO. A report from the UK Department of Health (1991) determined

a value for the lower reference nutrient intake (LRNI), which was established as the amount necessary to prevent goitre in adults (70 µg/day). The Reference nutrient intake (RNI) was then set at twice that value, rather than based on the amount required to meet the needs of 97.5% of the population, as the true RNIs were calculated.

IODINE INTAKE RECOMMENDATIONS BY AGE (MG/D)

	WHO (2007)	UK department of Health (1991)
INFANTS (<12 MO)	90 (0–24 mo)	60 (7–12 mo)
CHILDREN	90 (2–5 y) 120 (6–10 y)	70 (1–3 y) 100 (4–6 y) 110 (7–10 y)
	120 (11–12 y) 150 (13–18 y)	130 (11–14 y) 140 (15–18 y)
ADULTS (>18 Y)	150	140/150*
PREGNANCY	250	140/200*
LACTATION	250	140/200*

Table 1. 1Recommendations for iodine intake (µg/d) by age group

*BDA & *European Food Safety Authority (EFSA) recommendations*

1.2.5 Dietary sources of iodine

Milk and dairy products are the primary sources of dietary iodine intake in most Western countries, including the UK(Phillips, 1997, Dahl et al., 2004, van der Reijden et al., 2017b, Coneyworth et al., 2020). However, the iodine content in these foods is influenced by various factors, such as drinking water and animal feed fortification (Coneyworth et al., 2020, Flachowsky et al., 2014, Stevenson et al., 2018). In fact, the iodine content in food sources can vary based on the iodine concentration in the soil where plants are grown or animals are raised (Zhu et al., 2003).

In the past, there was a concern in the UK about the potential for excessive intake of iodine among individuals who consumed large amounts of milk. To address this concern, in the 1980s and 1990s, the Ministry of Agriculture Fisheries and Food (MAFF) monitored the levels of iodine in milk produced in

the country and estimated the exposure of different age groups to iodine(Witard et al., 2022). The regulation for the permitted iodine content in cattle feed has been modified since then, in order to avoid the potential toxicity of high levels of iodine in milk. According to the report from The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) in 2013, the recommended maximum concentration of iodine in cattle feed was reduced from 40 mg/kg dry matter to 5 mg/kg.

The values in Table 1.2 were obtained from the British Dietetic Association (BDA), which serves as a reliable reference for the iodine content of food consumed in the United Kingdom. Additionally, in aquatic environments, iodine is naturally incorporated into organisms, particularly marine organisms, at relatively high concentrations. Seafood, including marine-origin fish and crustaceans, as well as seaweeds and sea vegetables, is widely acknowledged to be the richest source of iodine in the human diet, and it also provides other crucial nutrients that promote human health and growth(Küpper et al., 2008, Palmieri and Forleo, 2020). In the table provided by BDA not enough detail regarding the variance on iodine content between fish species. A recent study done by Sprague et al. (2021) have analysed the iodine content in over 90 wild and farmed fish species available to UK consumers in which detail has been gathered in Figure 1.3. This analysis reveals a significant diversity in iodine concentrations, varying by farming methods (farmed or wild captured) and between different species of fishes. Given the current NHS recommendations consuming two portions of fish per week. The range of iodine levels found in these fish species could lead to substantial fluctuations in dietary iodine intake, underlining the complexity of ensuring adequate iodine intake through fish consumption.

Table 1. 2 **Estimated iodine content of foods in UK**

Name of Product	**Portion size**	**Iodine content(µg)**
Milk		
Cow's milk	200ml	50-100
Yoghurt	150g	50-100
Fish		
Haddock	120g	390
Cod	120g	230
Plaice	130g	30
Salmon fillet	100g	14
Canned tuna	100g	12
Shellfish		
Prawns	60g	6
Scampi	170g	160
Others		
Eggs	1 egg(50g)	25
Meat/poultry	100g	10
Nuts	25g	5
Bread (all types)	1 slice(36g)	5
Fruit and vegetables	1 portion (80g)	3

Source: British Dietetic Association The Association of UK Dieticians, Food Fact Sheet-Iodine, Nov 2022 (https://www.bda.uk.com/resource/iodine.html)

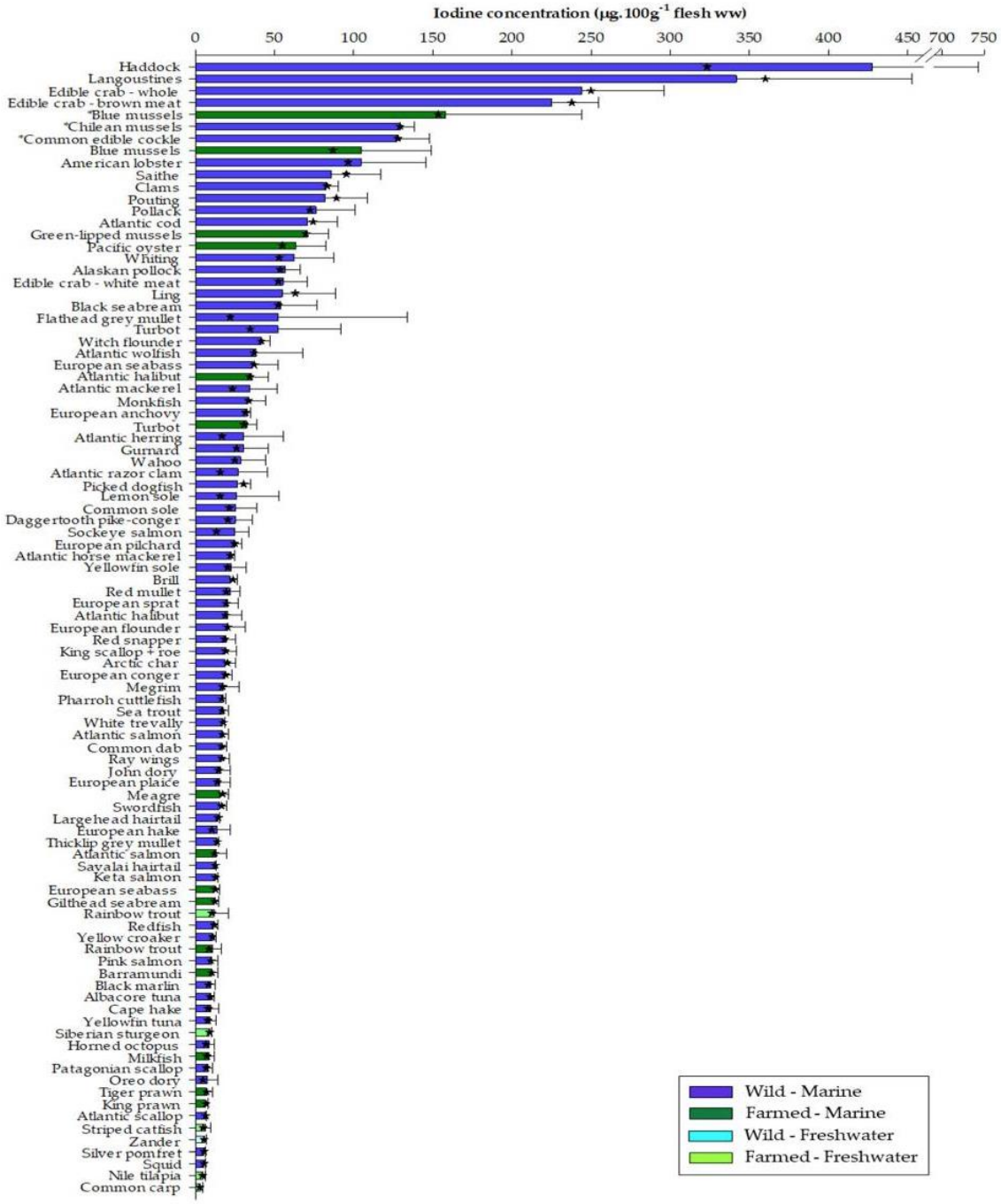


Figure1. 3 **Iodine concentrations of seafood samples**

Source: Sprague, M., Chau, T. C., & Givens, D. I. (2022). Iodine Content of Wild and Farmed Seafood and Its Estimated Contribution to UK Dietary Iodine Intake. *Nutrients*, *14*(1), 195. https://doi.org/10.3390/nu14010195

1.2.6 Assessment of iodine status

Several techniques are recommended for evaluating the iodine nutrient status in populations. The most common method for evaluating iodine status of a population is urinary iodine concentration (UIC), although the goitre rate and serum TG concentration and serum thyroid-stimulating hormone (TSH) are also options. The goitre rate suggests long-term iodine insufficiency (years), TG indicates thyroid activity and demonstrates the response within weeks, while UIC is a sensitive measure of recent daily iodine consumption (Zimmermann, 2009, Organization, 2007).

The majority of ingested iodine is eliminated through urine, making the Urinary Iodine Concentration (UIC) an effective measure of recent iodine intake (Zimmermann, 2008, Zimmermann and Andersson, 2012a). Despite the fact that the daily intake of iodine varies and can even differ throughout the day based on what has been consumed, it is still a reliable indicator of iodine status within a population (Als et al., 2000, Vejbjerg et al., 2009) The median UIC is the most frequently used method for evaluating the iodine levels of a population. In the report published with WHO(2013) indicate that when the iodine concentration is measured with urine samples, commonly using one-spot urine samples collected in the morning or other spot collections. Both spot urine and 24-hour urine samples are commonly used to evaluate the iodine levels in the general population. Although 24-hour urine collection is the ideal choice for determining the analytes of 24-hour iodine excretion, the acceptance of the 24-hour urine sampling protocol is usually low (Garde et al., 2004). Spot urine samples are often used as an alternative because they are easy to collect and cause minimal inconvenience to participants. In addition, addressing the variability in urine volume and iodine concentration is crucial for the correct interpretation of UIC. As suggested by Vejbjerg et al. (2009), standardised creatinine-iodine concentrations can mitigate this issue.

Various techniques, including paired-ion reversed-phase high-performance liquid chromatography (HPLC) and inductively coupled plasma mass spectrometry (ICP-MS)(Jooste and Strydom, 2010), have been established for

the detection of iodine levels in urine. Additionally, the Sandell-Kolthoff method has been widely adopted and is considered to be a reliable approach(Markou et al., 2002).

According to current standards, the ICP-MS technique is considered the most precise and has been designated as the official method by the Centers for Disease Control and Prevention (CDC)(Caldwell et al., 2005). On the other hand, the Sandell-Kolthoff method, a comparatively uncomplicated colourimetric approach utilising commonly available equipment, has shown a satisfactory correlation with ICP-MS measurements. However, the WHO still endorses the use of the Sandell-Kolthoff method for conducting epidemiological studies because of its simplicity and cost-effectiveness, which also produces minimal hazardous waste. Nevertheless, population-level estimates of iodine intake require a relatively large sample size (> 300) when using this method(Haap et al., 2017).

The standard for assessment for iodine (UIC) nutrition status in populations are shown in table 1.3.

1.2.7 Policy of iodine fortification and global iodine status in recent years

The Iodine Global Network (IGN) gathers information from UIC research carried out worldwide and continuously tracks the iodine status worldwide. In addition, the IGN gathers the national median UIC, mostly for school-aged children. The International Child Development Steering Group identified iodine deficiency as one of the four major global risk factors for poor child development (Walker et al., 2007). To increase population-level iodine intake and avoid IDD, salt iodisation has been introduced in many countries worldwide, as shown in Figure 1.4. Figure 1.4 shows the iodised salt coverage published by Unicef (2017) that 86% of households worldwide have access to iodised salt. According to UNICEF, this number has increased to 89% on their website since June 2022.

Median Urinary Iodine (µg/L)	Iodine intake	Iodine status
School-age children (6yrs or older)		
<20	Insufficient	Severe iodine deficiency
20-49	Insufficient	Moderate iodine deficiency
50-99	Insufficient	Mild iodine deficiency
100-199	Adequate	Adequate iodine intake
200-299	Above requirements	Slight risk of more iodine intake than adequate
>300	Excessive	Risk of adverse health consequences
Pregnant women		
<150	Insufficient	
150-249	Adequate	
250-499	Above requirements	
>500	Excessive	
Lactating women		
<100	Insufficient	
>100	Adequate	

Table 1. 3**The standard for assessment for iodine nutrition status in different population groups on UIC**

Although the specific programs differ based on the circumstances in each country, salt iodisation is a safe, economical, and successful method that is compulsory in 128 of the 196 countries around the world. Currently, 89% of families worldwide use iodised salt, compared to the report published in 2012 by Zimmermann and Andersson (2012b), as approximately 70% of households have access to iodised salt. As a result, there are now only 24 countries with IDDs, according to the iodine status guide published by Unicef (2018), down from 113 countries in 1993 and 39 countries in 2012.

However, issues with sustainability have taken the centre stage in nations that have recently implemented iodised salt programs. For example, Vietnam and Cambodia are recent examples of nations where program quality has declined (Darussalam and Excessive, 2020). Indeed, Zimmermann and Andersson

(2021) claim that ten of the 15 nations with the highest rates of stunted growth, where chronic undernutrition creates more than 38% of preschoolers to be stunted, are iodine-sufficient at the national level (Afghanistan, Chad, Democratic Republic of the Congo, Guatemala, Malawi, Niger, Papua New Guinea, Sudan, Timor-Leste, and Yemen). Only three of these nations (Burundi, Mozambique, and Madagascar) still do not have sufficient iodine in their national reports. Governments and the salt-related industry need to commit over a lengthy period to the salt iodisation program to improve the IDD within their nation.

In contrast, China has implemented a mandate of universal salt iodisation (USI) at the national level for more than 20 years. Although IDDs are effectively controlled, the risk of excessive iodine consumption remains controversial. According to the Sixth Tone (Wang, 2018), China is dropping its nationwide requirement that all iodised salt be in favour of regionally specific requirements. Although China's booming logistics sector and improvements in quality-of-life standards have helped to some extent close the gap between coastal and inland diets, The Sixth Tone notes that iodine intake still varies greatly across the nation due to dietary habits, water iodine levels, and individual's physical needs. The new regulation would authorize provinces with adequate iodine supplies to market non-iodized salt and encourage provincial governments to create iodized salt concentration criteria that take into account regional considerations like diets high in seafood and drinking water.

In comparison, as mentioned in 1.2.5, industrialised countries, that mainly use milk as an iodine source rather than rely on iodised salt, such as Italy(Pastorelli et al., 2015), Australia(Li et al., 2006), Canada(Borucki Castro et al., 2010), Poland(Śliwiński et al., 2015), Denmark(Rasmussen et al., 2008), the UK(Dineva et al., 2021) and the US(Pearce et al., 2004), or country like Japan with a diet habit containing sufficient iodine (seaweed) on their dinner table would have different health issues regards to their iodine status. This is because dairy cows have a broad range of milk iodine concentrations (MIC) in the milk produced. in 1.2.3, we specifically stated that concerns in the

UK about the potential for excessive intake of iodine changed the iodine content added to cattle feed. In addition, according to research by Zimmermann and van der Reijden et al. (2017a), MIC ranges of 33-534 µg/L and typical MIC in the range of 100-300 µg/L have been reported in recent studies published since 2000, mostly from industrialised countries.

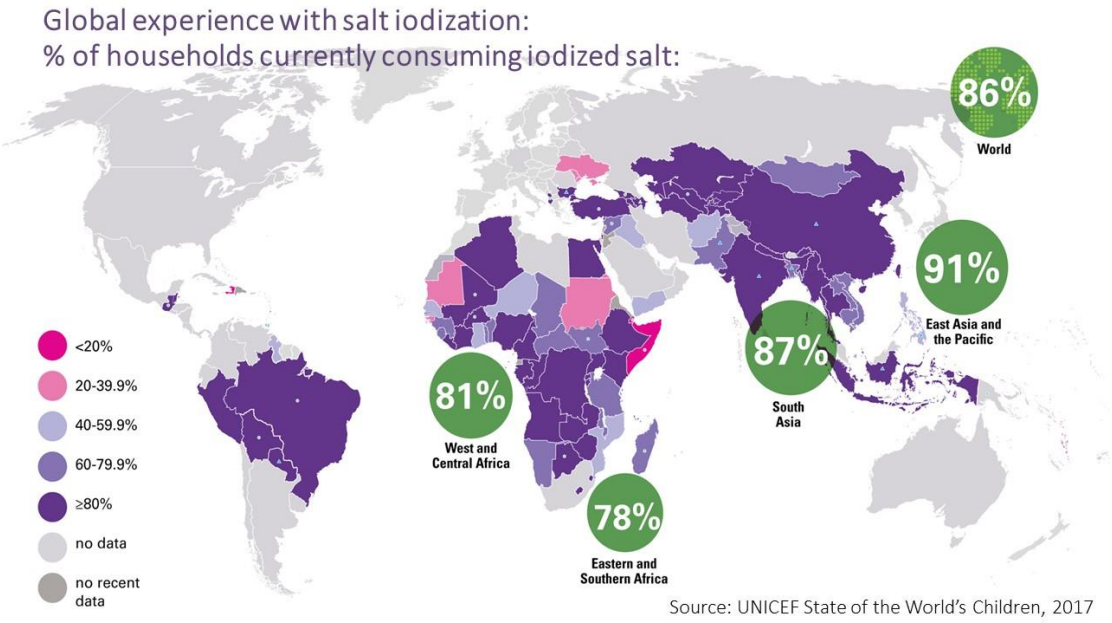


Figure1. 4 **Percentage of households consuming iodized salt.**

The global map of iodized salt coverage, with East Asia and the Pacific have the most coverage among households (91%), and Eastern and Southern Africa had the lowest coverage at 78%. Figure 1.8 was taken from IGN, Sources from UNICEF in 2017.

The global scale of iodine status posted by the IGN in 2021 indicate that from the perspective of the national level, 13 nations have reached an adequate level, as determined by their sub-national data. National data from 105 countries also show adequate iodine levels (Figure 1.5). Additionally, nine and four countries, as shown in red, supported by national or sub-national data, had excess UIC levels. Apart from 42 countries that did not provide any data, there are 18 countries' national UIC levels below the sufficient line, as well as three countries with sub-national data and an insufficient UIC level.

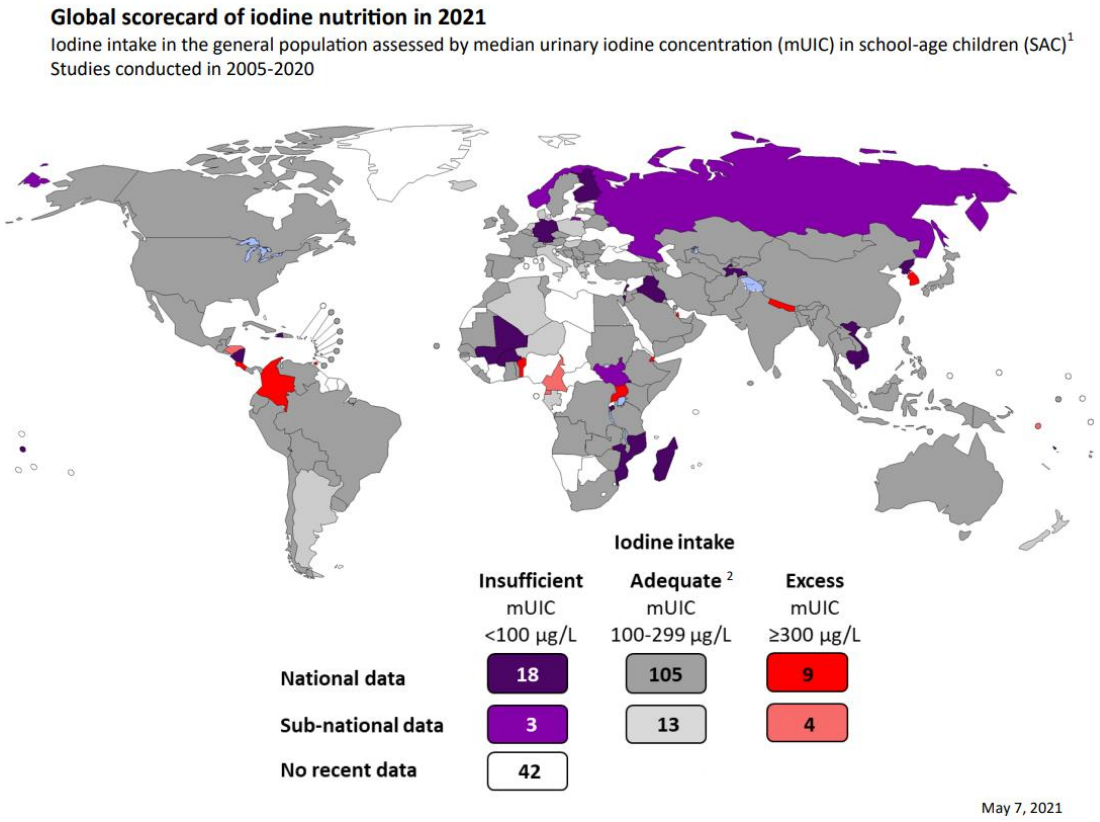


Figure1. 5 **Global iodine status in 2021**

Based on data from studies done between 2005 and 2020, the national median UIC in school-age children was used to estimate the iodine intake in 194 WHO Member States in 2021. insufficient: National/Sub-National/No recent data: Number of countries; Median UIC 100 µg/L; Appropriate: Median UIC 100-299 µg/L; Excess: Median UIC 300 µg/L. Urinary iodine concentration, or UIC. Figure was taken from IGN (2021).

1.2.8 Iodine status in UK

From a historical perspective, a survey conducted in 1924 by school medical officers on behalf of the Board of Education in England and Wales assessed the iodine deficiency situation in the UK. The survey focused on 12-year-old schoolchildren. The recruitment of 375000 children revealed a high prevalence of visible goitre (up to 30% in some communities), which emphasises the need for a national iodisation program. This survey is considered a significant landmark, as it helped identify the geographic distribution of the disease. In addition, during the same decade, research

showed that iodine supplementation of livestock could improve their reproductive performance, which subsequently resulted in the use of iodine-enriched cattle feed (marketed by spillers in 1937). It has been observed that an increase in iodine levels in milk, particularly during the winter months, can occur when pasture is in short supply and cattle are forced to rely on iodine-rich feed (Phillips, 1997). This increase in milk availability through the Milk Act (1934) and the National Milk Scheme (1940) greatly boosted milk consumption, as it provided free or subsidised milk for expectant mothers and young children. In the early 1950s, the average milk consumption had significantly increased. In winter milk consumption alone achieve the daily intake of 150 µg/day but summer (Phillips, 1997). The WHO stopped collecting data from the United Kingdom on a regular basis due to the belief that iodine deficiency had been completely eliminated. Detail of iodine content policy changes also addressed in previous section 1.2.5.

In recent years, it had been widely believed that iodine deficiency only affected developing countries and areas with iodine-deficient soil, leading to the promotion of iodination of salt in such regions. However, recent findings have shown that iodine insufficiency is prevalent in the UK and other European countries, especially in more vulnerable groups such as children and women of reproductive age, despite the previously held belief of sufficient iodine levels in these countries (Bath et al., 2013, Bath et al., 2014, Andersson et al., 2007). A study conducted in the UK in 2009 on the iodine levels of 859 14-15-year-old girls revealed that 51% of the sampled population had mild deficiency and 17% had moderate to severe iodine deficiency (Vanderpump et al., 2011). As a result of these studies, UK data are now collected and UIC has been measured as part of the National Diet and Nutrition Survey (NDNS) since 2013.

1.2.9 Iodine status with Vegan/Vegetarian

Recently, the popularity of vegetarian and vegan diets has increased. More individuals opted to consume only non-animal products. A vegan diet specifically excludes eggs, dairy, red and white meat, and all fish types. Subcategories of vegetarianism include pesco-vegetarianism, which permits

the consumption of fish, and lacto-ovo-vegetarianism, which permits the consumption of milk and eggs (Phillips, 2005). Each group within the vegan and vegetarian populations had its own set of limitations for food choices.

The consumption of vegetarian and vegan diets varies worldwide. In developing countries, people often choose to follow a vegetarian diet because of various factors, such as religious beliefs, societal norms, or financial limitations, rather than their personal preferences(Sebastiani et al., 2019). People in developed countries generally have the opportunity to decide what they eat and the level of dietary restrictions to which they adhere. These diets can also be chosen for reasons such as concern for the environment, personal beliefs about ethics or religion, maintaining good health, and social motivations. Fast-food and conventional restaurants also offer animal-free products aimed at attracting customers and adding item indications to their menus that cater to various dietary restrictions(Dunn et al., 2021). There are concerns about the ability of such diets to supply important micronutrients, such as iodine (SACN, 2014).

A study conducted by Eveleigh et al. (2023) reported in the review included 11 articles confirming that individuals on vegan and vegetarian diets, residing in countries without mandatory salt iodisation, and not consuming seaweed or iodine-containing supplements, are still at an increased risk of low iodine status, iodine deficiency, and inadequate iodine intake compared to less restrictive dietary groups. The median UIC of all the dietary groups studied in this review was below the WHO criteria for optimal iodine status (100–200 µg/L).

Hence, lower levels of essential nutrients have been observed in individuals who follow a long-term vegan diet, particularly iodine. This highlights the importance of providing nutritional guidance to the vegans. Additionally, since vegans and vegetarians actively avoid iodine-rich items (fish, shellfish, milk, and dairy products) in their diet, but rely on seaweed and supplementation for iodine intake, there is a greater level of fluctuation in their iodine consumption. This was mainly due to the high levels of iodine present in these sources. It

may also create thyroid-related health issues against the idea of a healthy diet for vegan and vegetarian populations.

1.2.10 Trends and patterns of iodine intake fluctuation

Defining the fluctuation of certain micronutrients in the human body is a challenging task, primarily because of irregularities in our eating habits. The iodine content of daily food intake is inconsistent and varies in terms of both quantity and quality. As a result, fluctuations in micronutrient levels are bound to occur. This is further compounded by sudden changes in dietary choices, such as switching from a typical diet to a vegan or vegetarian diet. as mentioned in the previous section, the vegan/vegetarian population actively avoids iodine-rich food and combines that with a modern lifestyle, frequent meal skipping, on-the-go eating, and irregular mealtimes. The daily intake may fluctuate significantly as iodine-rich foods are generally scarce in such diets unless supplements are taken. Moreover, individuals who have immigrated from one country to another may experience considerable changes in their iodine status because the availability and accessibility of iodine (iodine rich food) vary across different regions (i.e. from country with USI to country that does not). When the region that full initiation of USI is not possible then the administration of massive doses of iodine in the form of slowly resorbable iodised oil which is also the form of fluctuation.

Consider Japan as a case study for iodine fluctuation, where more than 20 species of red, green, and brown seaweeds are incorporated into their meals (Arasaki and Arasaki, 1983). Daily iodine consumption in Japan is estimated to be between 1,000-3,000 μg, which has been associated with health benefits not observed in other cultures(Zava and Zava, 2011). The Japanese have a low incidence of specific types of cancer, such as breast cancer(Mettlin, 1999), and are renowned for their longevity (WHO,2010). Nonetheless, the iodine content of seaweed varies depending on the species, and individual consumption habits in Japan differ, leading to fluctuating iodine intake.

Although it is commonly believed that excessive iodine consumption does not lead to thyroid antibody positivity, it is important to note that high iodine intake can exacerbate or trigger symptoms in individuals with preexisting thyroid autoimmunity or other underlying thyroid problems (Nagata et al., 1998). Transient hypothyroidism and iodine-induced goitre are common in Japan and can be reversed by limiting seaweed consumption (Ishizuki et al., 1989, Nishiyama et al., 2004, Konno et al., 1993). As there is no USI in Japan, restricting seaweed intake helps stabilise iodine intake levels.

Limited research has addressed the fluctuations in iodine intake and their potential health consequences. Studies such as that conducted by Seibold-Weiger et al. (1999) analysed the longitudinal iodine concentration in breast milk from mothers of preterm infants with and without iodine supplementation. They noted that variations in iodine concentrations in breast milk could be attributed to fluctuating daily iodine intake. Additionally, a study by van de Ven et al. (2014) involving 980 participants observed an ongoing decrease in TSH and an increase in FT4 levels in a population previously deficient in iodine, despite currently having adequate iodine status. This suggests that low iodine intake early in life can lead to thyroid autonomy, which persists even with normal iodine intake later in life, potentially increasing the risk of hyperthyroidism. Additionally, Mian et al. (2009) highlighted that iodine deficiency was prevalent among 322 pregnant women, particularly among foreign women, with cow's milk being their primary iodine source.

These analyses provide valuable insights into the broader implications of iodine intake fluctuations and underscore the need for public health strategies to address these concerns. However, there is a significant gap in mechanistic studies of iodine fluctuation. Such research could enhance our understanding of the implications of iodine fluctuations on thyroid-related health issues, leading to more informed public health policies and intervention strategies.

1.3 Integrated Perspectives on Thyroid Physiology

1.3.1 General information of Thyroid

Thyroid gland anatomy and location.

The thyroid gland is an endocrine organ consisting of two connected lobes, that is located at the front of the neck in humans. The lobes of the thyroid are connected by a thin band of tissue known as the thyroid isthmus, that connects the lower two-thirds of the lobes. The thyroid represents the largest endocrine gland in humans and weighs about 1 to 2 g at birth and between 10 to 20 g in adults.

The functional structure of the thyroid gland is distinctive among all other endocrine glands in the human body since it is composed of different sizes of ball-like follicles and has interfollicular connective tissues with blood capillaries and parafollicular cells. Each follicle is composed of a large number of thyroid follicular epithelial cells that form a simple epithelium around a lumen. Each follicular lumen is a completely enclosed area in which thyroglobulin-rich colloid is present as shown in Figure 1.6, (Fujita, 1975).

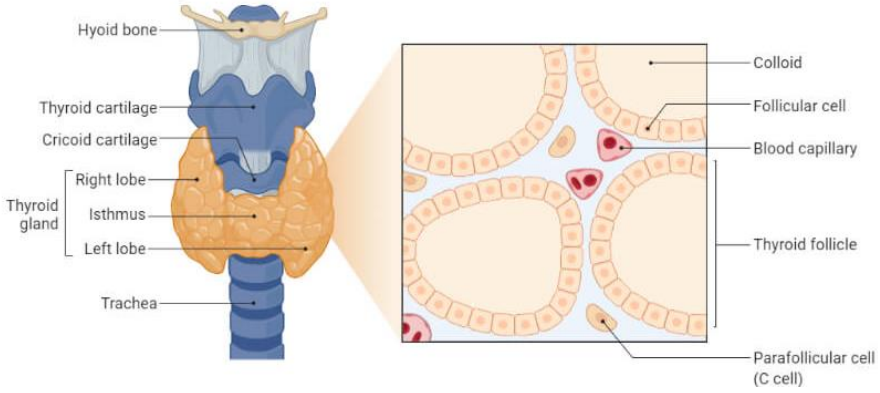


Figure1. 6 **The location and structure of human thyroid gland**

An overview of the human thyroid gland with the cross-section detail with it. Figure is created with *BioRender.com.*

General functions of the thyroid gland in endocrine system.

The thyroid gland is one of the main regulators of metabolism. The most important function of the thyroid gland is to produce thyroid hormones, T3 and T4. Metabolic processes increased by thyroid hormones include: Basal Metabolic Rate, Gluconeogenesis, Glycogenolysis, Protein synthesis, Lipogenesis, Thermogenesis(Oppenheimer et al., 1991, Moini et al., 2020).

1.3.2 Biosynthesis of Thyroid Hormones

As mentioned in 1.2.2, the production of TH rely on the presence of iodine. The thyroid gland utilizes iodide in a complex process to produce thyroid hormones. It starts with iodide being transported from the capillary to the follicular cell of the thyroid via an active transport system. This occurs against electrochemical sodium gradients through the sodium/iodine symporter protein (NIS) located in the basolateral membrane of the follicular cell (Figure 1.7). The energy required for this process is linked to the ATPase-dependent Na+-K+ pump (Dai et al., 1996, Kopp et al., 2015).

The second step of TH synthesis is the synthesis and secretion of TG, which occurs independently in thyroid follicular cells. TG serves as a substrate of TH, is produced on the rough endoplasmic reticulum (ER), and then moves to the Golgi apparatus before moving toward the apical membrane of the follicular cell. TG is released into the follicular lumen via exocytosis(Di Jeso and Arvan, 2016).

After iodide is taken up by the thyroid cell, it moves towards the apical side to enter the follicular lumen, which is mediated via a sodium-independent iodide/chloride transporter called pendrin (SLC26A4, PDS)(Braverman and Cooper, 2013). Iodide (I-) must be oxidised to a higher oxidation state (I2) before the next step; hence, H2O2 and Thyroid peroxidase (TPO) are needed to catalyse it.

This is followed by organification of thyroglobulin (TG), which is also stimulated by H2O2 and TPO, where iodine attaches to tyrosyl residues within TG to produce the TH precursors DIT and MIT. The next step is the coupling reaction, whereby two DIT molecules couple to form T4, and one MIT molecule couples with one DIT molecule to form T3(Braverman and Cooper, 2013).

TG which is still bound to THs (T4 and T3), is stored in the follicular lumen until required. It then undergoes a process called endocytosis to enter the thyroid cells, as it moves towards the basal membrane, possibly by

microtubule and microfilament function(Ahad and Ganie, 2010), which then interacts with the lysosome, as lysosomal proteases digest TG and release THs (T3 and T4) into the circulation. While undergoing this procedure, MIT and DIT are also released into the cytoplasm, where they are subsequently recycled through transmembrane protein Iodotyrosine dehalogenase 1 (DEHAL1), resulting in a continuous cycle of iodide utilisation within the thyroid (Gnidehou et al., 2004).

1.3.3 Thyroid Hormone Transportation

Mechanisms of thyroid hormone transport in the bloodstream.

Both T3 and T4 are classic endocrine hormones that are released into the bloodstream upon production in the thyroid gland via binding to transport proteins due to their hydrophobic nature. Three major proteins, thyroxine-binding globulin (TBG), transthyretin (TTR), and albumin (ALB), are responsible for carrying THs to reach the target site. Only a small proportion (approximately 0.03% of T4 and 0.3% of T3)(Braverman and Cooper, 2013) of thyroid hormones are active and unbound to transport proteins; these can be identified as free T3 and T4 (fT3 and fT4).

It seems logical and reasonable to assume that one of the functions of TH-binding proteins in the serum is to protect the body from the effects of sudden changes in hormonal secretion to achieve homeostasis. Based on the research done by Chan et al. (1972), the TH binding protein also likely functions as a further defense against iodine loss by giving the macromolecular characteristics of small iodothyronine molecules, which reduces their urinary loss. Regarding their relative abundance, ALB is present in serum at a molar concentration approximately 100 times that of TTR and 2,000 times that of TBG (Table 1.4). However, from the perspective of T4 association constants, TBG has the highest affinity, which is 50 times greater than TTR's and 7,000 times greater than ALB's.

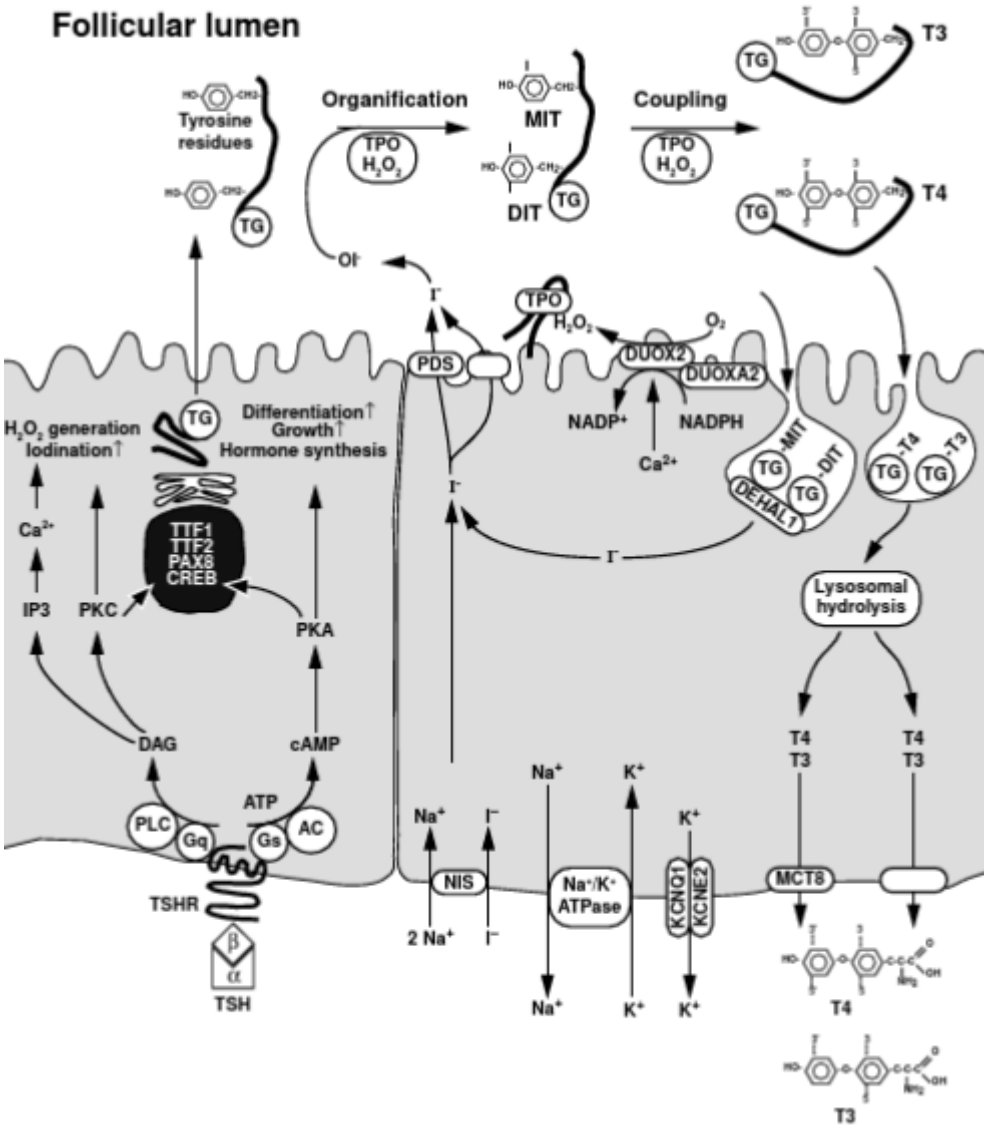


Figure1. 7 **Thyroid hormone synthesis, secretion, and major signaling pathways in thyrocytes**

AC, adenylyl cyclase; cAMP, cyclic AMP; DAG, diacylglycerol; DEHAL1, iodotyrosine dehalogenase 1; DIT, diiodotyrosine; DUOX, dual oxidase 2; DUOXA2, dual oxidase maturation factor 2; KCNQ1 and KCNE2, potassium channel subunits; MCT8, monocarboxylate transporter 8; MIT, monoiodotyrosine; NIS, sodium/iodide symporter; PDS, pendrin (SLC26A4, solute carrier 26A4); PKA, protein kinase A; PLC, phospholipase C; TG, thyroglobulin; TPO, thyroid peroxidase; TSH, thyrotropin; TSHR, TSH receptor. Figure was taken from Braverman and Cooper (2013) Werner & Ingbar's The Thyroid: A Fundamental & Clinical Text, 10th Edition Section1.Chapter 4B.Thyroid Hormone Synthesis

As shown in the table, TBG is the major thyroid hormones binding protein; after being produced from hepatocytes, TBG is responsible for binding 75% of both T3 and T4 in plasma. TBG has one binding site for THs and a half-life of approximately five days (Refetoff, 2000). Despite its major role in thyroid hormone binding, excess, deficiency, and functional changes in TBG do not change or cause impairment of thyroid function. However, because TBG is the main carrier of THs in humans, any changes in the circulating concentration of TBG have a direct effect on the circulating concentration of THs. The plasma concentration of TBG decreases in hyperthyroidism and increases in hypothyroidism.

TTR and ALB play a minor role as binding proteins for carrying THs through the bloodstream. Although both have multiple binding sites for THs (two for TTR and four for ALB), they also take the role of transport other proteins that are important for living organisms. Generally speaking, in mammals, transthyretin binds T4 with a higher affinity than T3 (20% and 5%, respectively), and ALB has a higher affinity towards T3 than T4 (5% and 20%, respectively), as shown in Table. TTR acts as the major carrier in the central nervous system and carries retinol into the bloodstream. In addition to TH, TTR is indirectly linked to the transport of vitamin A via retinol-binding protein (RBP)(Raz and Goodman, 1969). Despite having four iodothyronine-binding sites on its molecule, ALB has only one that has a high affinity for T4 and T3, and its binding site is 10,000 times less effective than that of TBG(Tabachnick and Giorgio, 1964). Makes the contribution of ALB to the transport of thyroid hormones is minor.

	TBG	TTR	ALB
Molecular mass(kDa)	54	55	66.5
Half-life (Days)	5	2	15
Number of Binding sites	1	2	4
% Bound T4	75	20	5
% Bound T3	75	<5	20
Concentration in serum(mg/liter)	16	250	40,000
Other ligands	None	Retinol-binding protein	Steroids, Vit D, ions, lipids, drugs, etc.

Table 1. 4 **Main characteristics of the major thyroid hormone transporters in humans.**

Reinvented from the thyroid chapter 5 thyroid hormone transport proteins, Braverman and Cooper (2013).

Mechanisms of thyroid hormone transport into target cells.

Upon arrival at the target cells, THs can dissociate from their binding proteins and enter the cells via carrier-mediated transport. The process of transporting THs into the target cell was initially considered diffusion due to the lipophilic nature of THs(Visser et al., 2011). In the 70s, published evidence suggested that thyroid hormones crossing the plasma membrane require a carrier-mediated mechanism. However, the role of passive diffusion in this process remains limited(Hennemann et al., 2001). In addition, studies with successful discoveries have been established with different models that study the role of thyroid hormone transporters and understand their tissue distribution, the contribution of thyroid hormone transporters to different thyroid hormones, and the regulation of TH levels in the target tissue after the millennium. Monocarboxylate transporter 8 (MCT8), MCT10, organic anion transporting polypeptide (OATP) 1C1, and SLC17A4 have recently been identified as the most effective thyroid hormone transporters(Kinne et al., 2011, Groeneweg et al., 2019, Groeneweg et al., 2022). The transport of THs into target cells is

mediated by carrier proteins, as shown in table 1.5, that facilitate their movement across the plasma membrane. These transporters are essential for the uptake of THs into the target tissues and play a significant role in the regulation of TH levels in the target tissue(Kinne et al., 2011, Groeneweg et al., 2019, Groeneweg et al., 2022).

Conversion of T4 to T3 in peripheral tissues and organs via Diodinases(DIOs).

When THs reach their intended destination, they can detach from their binding proteins and gain entry into cells via carrier-mediated transport(Friesema et al., 2005). Once inside target cells, the deiodinase family of enzymes controls the activation and inactivation of THs. To gain the full bioactivity of TH, T4 must be activated by deiodination to remove the outer ring iodine (5' position) to convert it to T3 (Figure 1.8). In addition, T4 can be converted to its inactive form by deiodination of the inner tyrosyl ring (5 position). Three deiodinases, types 1, 2, and 3 iodothyronine deiodinases (DIO1,2&3, respectively), were identified(St Germain et al., 2009). They differ in tissue distribution and catalytic characteristics and play a key role in regulating TH homeostasis. Despite the similar roles of DIO1 and DIO2, they both have the ability to remove one iodine atom from T4 to produce T3. As shown in Table 1.7, DIO1 is localised to the cell membrane, whereas DIO2 is localised to the ER membrane (Baqui et al., 2000) . Both DIO1 and 2 have an active centre in the cytosol, which primarily produces T3 for local use upon entering cells, while DIO3 molecules appear to protrude towards the extracellular area(Sabatino et al., 2021). For example, as shown in Figure 1.8, T4 is deiodinated by DIO2 to produce the active form of thyroid hormone T3. DIO3, on the other hand, prevents T4 and T3 from activating as it produces reverse T3 and T2. Furthermore, DIO1 can activate or inactivate T4 by generating both T3 and reverse T3 on an equimolar basis but with poor kinetic efficiency. T2, an inactive byproduct of both routes, is quickly digested by further deiodination (Bianco and Kim, 2006).

Transporter	Gene name	Tissue	Substrate uptake	Efflux
MCT8	SLC16A2	Developing and adult brain; vascular endothelial cells of BBB; choroid plexus; liver; kidney; thyroid; pituitary	T3, T4 > rT3 > T2	T3;T4
MCT10	SLC16A10	intestine, kidney, liver, skeletal muscle, heart, placenta, pancreas	T3 >T2 >> T4, rT3	T3
OATPs OATP1A1	SLCO1A2	Brain; endothelial cells of BBB; kidney, liver, lung, ciliary body, placenta	T3	nd
OATP1C1	SLCO1C1	Brain (glial and neuronal cells); BBB; choroid plexus testes (Leydig cells); ciliary body; adipose tissue	T4 > rT3 >T3	nd
LAT1	SLC7A5	Brain; placenta; testes; leukocytes; fetal liver; bone marrow	3,3'-T2 > rT3 > T3 > T4	3,3'-T2
LAT2	SLC7A8	Kidney; placenta; brain; spleen; prostate; testes; ovary; intestine, testis, ovary, liver, heart, skeletal muscle, lung, stomach	3,3'-T2 > T3	
LAT3	SLC43A1	Placenta, Liver, Kidney, Pancreas, Skeletal Muscle, Heart	None	MIT
LAT4	SLC43A2	Placenta, leukocytes, Skeletal Muscle, Spleen, kidney and heart	None	MIT
NTCP	SLC10A1	Placenta,	T4, T3	no
SLC17A4	SLC17A4	GI tract; liver; pancreas; kidney	T3; T4	nd

Table 1. 5 **Human thyroid hormone transporter**

n.d. not detected. BBB blood brain barriers; Table is reinvented from the research paper done by Kinne et al. (2011) and Groeneweg et al. (2019).

	DIO1	DIO2	DIO3
Subcellular location	Cell membrane	ER membrane	Cell membrane
Tissue location	Liver, Kidney, Thyroid	BAT; thyroid, heart, brain, sk muscle	CNS, skin, placenta
Substrate preference	rT3>T4>T3	T4>rT3	T3>T4
Half-life	>12hrs	40mins	12hrs

Table 1. 6 **Characteristics of Deiodinases**

Recreated from Braverman and Cooper (2013) and Sabatino et al. (2021)

BAT (Brown adipose tissue); CNS (central nervous system)

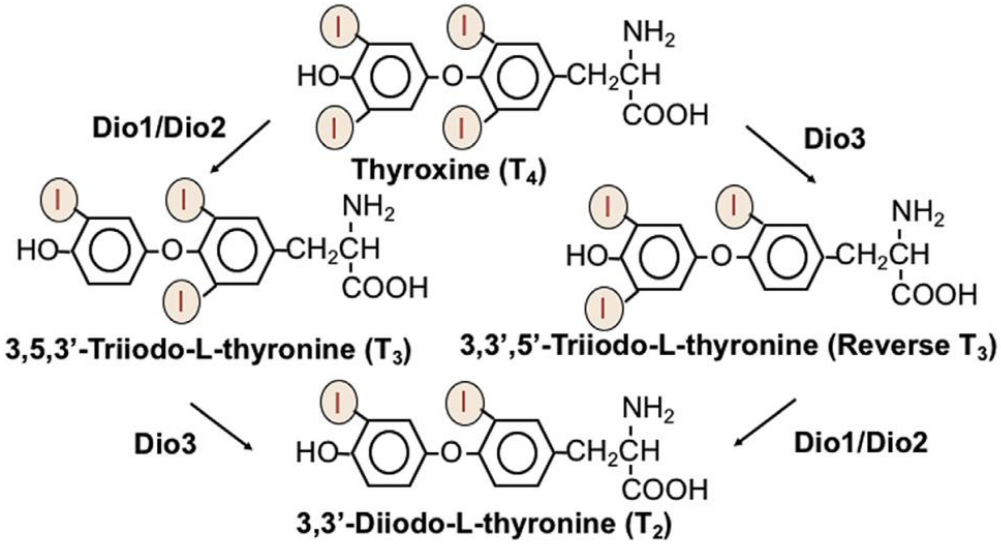


Figure1. 8 **Thyroid hormone and deiodinase.**

Deiodinase type 1 (DIO1) and Deiodinase type 2 (DIO2) are responsible to convert thyroxine (T4) to triiodothyronine (T3); Deiodinase type 3 (DIO3) is responsible for the inactivation of T4 to reverse T3 (rT3). Figure was taken from chapter 21 of Hormonal signalling in Biology and medicine; Liu et al. (2020)

1.3.4 Thyroid Hormone Action and Mechanisms

Mechanism of action of thyroid hormones at the cellular level.

As mentioned in Section 1.3.1, THs are critical regulators of cellular metabolism and growth. At the cellular level, these hormones exert their effects by binding to specific receptors, known as thyroid hormone receptors (TRs). TRs belong to the nuclear hormone receptor family(Flamant et al., 2006). TRs interact with specific DNA sequences called thyroid hormone response elements (TREs), located in the promoter regions of target genes (Figure 1.9). By binding to TREs, TRs regulate the expression of target genes and modulate their transcription by either increasing or decreasing their transcriptional activity.

There are two main types of TRs, known as alpha (TRα) and beta (TRβ), which are encoded by two separate genes: THRA on chromosome 17 and

THRB on chromosome 3. These genes produce various forms of TR proteins through a process called alternative splicing. (Chiamolera et al., 2012).

The distribution of specific isoforms varies across different tissues and species. In humans, TRα predominantly resides mainly in the heart, brain, and bone, while TRβ is predominantly found in the liver, kidneys, pituitary gland, and brain. TRβ plays a crucial role in regulating metabolic rate (Bryzgalova et al., 2008). Retinoid X receptor (RXR) is primarily known to function as a heterodimeric partner that binds to other nuclear receptors at DNA response elements. However, it can also be activated by ligand binding and has the ability to regulate gene expression. TR forms a complex with RXR, forming a heterodimer that binds to TRE, and this complex can either activate or inhibit the transcription of target genes (Mullur et al., 2014).

As shown in Figure 1.9, coactivators are recruited by the TR-RXR–TRE complex to stimulate the transcription of T3-regulated genes (Dasgupta et al., 2014). Corepressors collaborate with histone deacetylases (HDACs) to bring them to the promoter regions of target genes, thereby diminishing the transcription of T3-regulated genes and counteracting the effects of other nuclear receptor complexes(Hsia et al., 2010). The interplay between TR-RXR complexes, coactivators, and corepressors forms a highly regulated network that controls the transcriptional reaction to THs, underpinning their essential functions in cellular metabolism and growth.

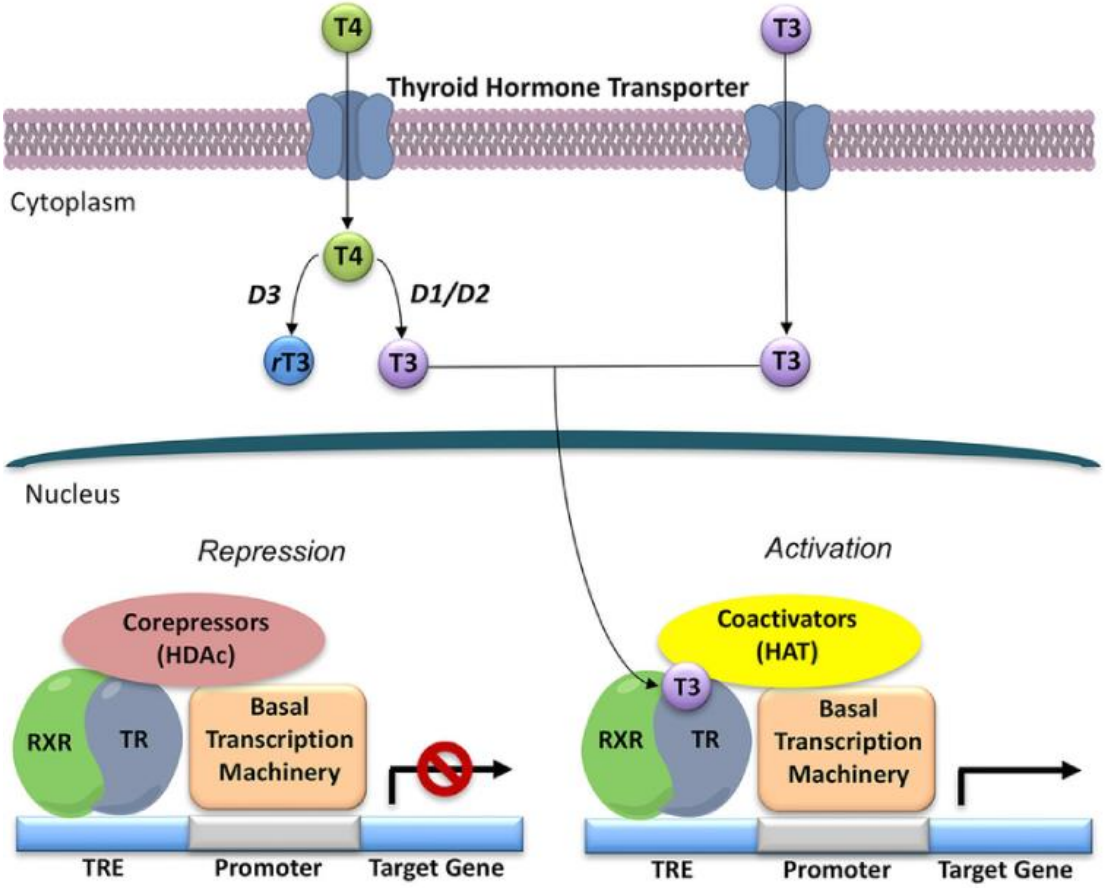


Figure1. 9 **General model for thyroid hormone (T3) action in the nucleus.**

The Thyroid Hormone Receptor (TR) acts as a transcription factor that regulates various TH target genes; bind to Retinoid X receptor (RXR), TRs interact with specific DNA sequences called thyroid hormone response elements (TREs), then recruits either corepressors and their associated histone deacetylases (HDACs) to repress target gene expression, or T3 binds to the complex that then recruits the coactivators and associated histone acetyltransferases (HATs) to promote target gene expression. Figure was taken from Saponaro Federica , Sestito Simona, Runfola Massimiliano, Rapposelli Simona, Chiellini Grazia. Selective Thyroid Hormone Receptor-Beta (TRβ) Agonists: New Perspectives for the Treatment of Metabolic and Neurodegenerative Disorders. Frontiers in Medicine. 2020. 10.3389/fmed.2020.00331

1.3.5 Regulation of Thyroid Function

Hypothalamic-Pituitary-Thyroid Axis

The thyroid gland is responsible for producing all thyroid hormones and is primarily regulated by the hypothalamic-pituitary-thyroid axis (HPT axis). This axis consists of various components, including the hypothalamus, pituitary gland, and thyroid gland, as shown in Figure 1.9. The production and secretion of TSH, which stimulates the thyroid gland, is regulated by the thyrotropin-releasing hormone (TRH) produced by the hypothalamus. TSH, in turn, stimulates the thyroid gland to produce T4 and T3, which are then released into the circulation in either a free or bonded form. The levels of T4 and T3 in circulation are regulated through a negative feedback loop, indicating that high levels of these hormones inhibit the secretion of TSH and TRH. This feedback is achieved through the activity of three deiodinase enzymes (DIO1, DIO2, and DIO3) that metabolize thyroid hormones (Braverman and Cooper, 2013, Mullur et al., 2014). A study conducted by Du et al. (2022)revealed that chronic iodine excess in Wistar rats led to elevated TSH levels but had no impact on TT3 and TT4 levels. Furthermore, prolonged high iodine intake results in decreased serum TRH levels in the hypothalamus.

Role of TSH in regulating thyroid hormone production.

TSH is the primary hormone that stimulates thyroid activity, as shown in Figure 1.10. TSH binds to the G protein-coupled TSH receptor (TSHR) on thyroid follicular cells, thereby stimulating the production and release. Additionally, other modulators such as IGF-1, inflammatory cytokines such as IL-1 and IL-6, and iodide availability in the system also play a role(Eggo, 2010).

In addition, NIS expression and function are tightly regulated by TSH, which utilises the cyclic adenosine monophosphate (cAMP) pathway to increase NIS activity in thyroid follicular cells (Riesco-Eizaguirre and Santisteban, 2006, Huang et al., 2011, Braverman and Cooper, 2013). Both in vivo and in vitro studies show that TSH up-regulates the mRNA and protein expression of NIS

(Levy et al., 1997, Kogai et al., 1997, Eng et al., 2001). Serum TSH is the primary screening test for thyroid disorders, both hypothyroidism and hyperthyroidism, as outlined in Section 1.2.6. This is because changes in TSH occur earlier than changes in T3/T4. If the TSH value falls outside the range of 0.4 to 4.5 milliunits per litre (mU/L), further testing of T3 and T4 should be performed. Nevertheless, in order to test for thyroid disorders, the TSH test is more reliable, as it is more dependable than measurements of plasma T3/T4 levels, which are prone to fluctuation(Sheehan, 2016).

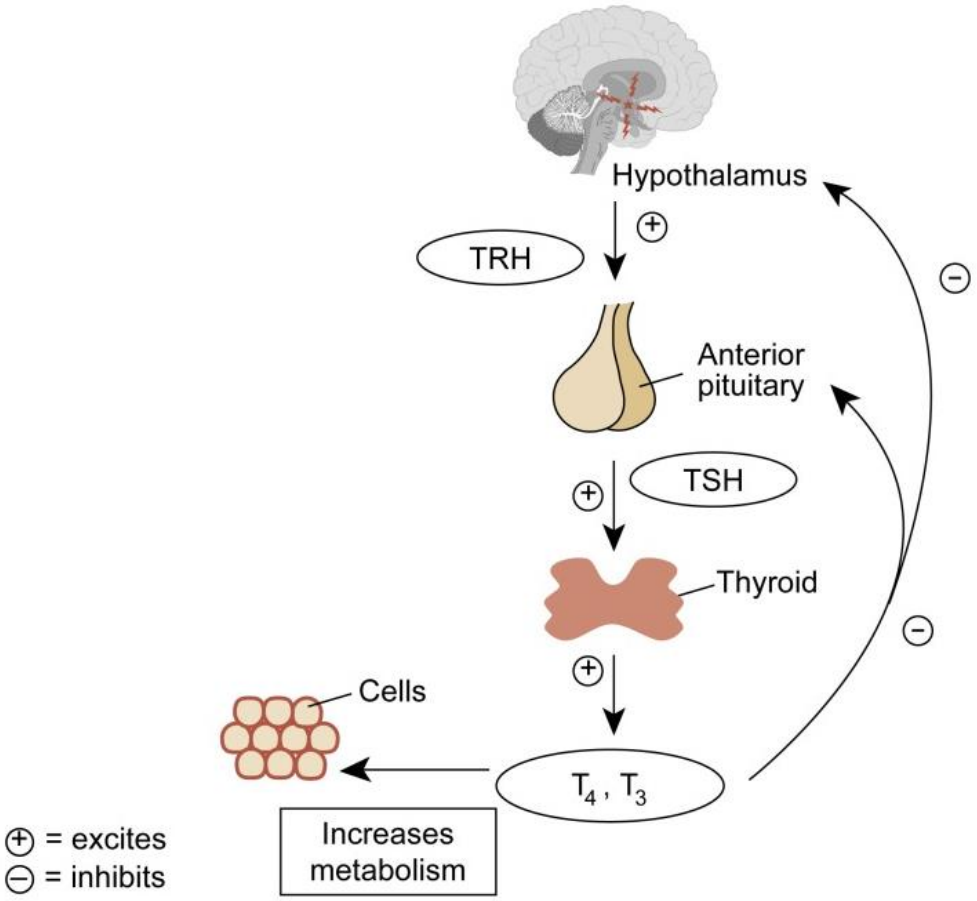


Figure1. 10 **The hypothalamic–pituitary–thyroid axis.**

Thyrotropin-releasing hormone (TRH) is produced and secreted by the hypothalamus and can stimulate the anterior pituitary cells to produce thyroid-stimulating hormone (TSH). TSH then stimulate the thyroid gland to secrete T4 and T3, the main function of which is to regulate cell metabolism. In order to reduce TRH and TSH secretion, T4 and T3 both feedback to the hypothalamus and pituitary. Figure was taken from Emanuele N, Emanuele MA. The endocrine system: alcohol alters critical hormonal balance. Alcohol Health and Research World. 1997 ;21(1):53-64.

1.3.6 Oxidative Stress and Thyroid Function

Cellular Stress and Reactive Oxygen Species

Reactive Oxygen Species (ROS), including peroxides (R-O-O-R) and superoxide (O2-), are highly reactive chemicals formed from oxygen. These molecules are by-products of normal cellular metabolism but can also arise from environmental stressors. Although ROS facilitate important cellular signalling processes, excessive ROS levels can lead to oxidative stress, adversely affecting cellular integrity (Zhang et al., 2016).

Living organisms contain various sources that produce ROS.

Mitochondria are the primary endogenous sources of ROS because of their role in energy production via oxidative phosphorylation(Ernster and Schatz, 1981). Peroxisomes, which contribute to fatty acid metabolism and detoxification (Del Río and López-Huertas, 2016), also produce various types of ROS, including hydrogen peroxide (H_2O_2) and hydroxyl radicals (-OH)(Di Meo et al., 2016). The ER, crucial for protein folding and lipid synthesis(Schwarz and Blower, 2016, Chong et al., 2017), can become a ROS source when protein misfolding occurs, leading to ER stress and the activation of the unfolded protein response (UPR), further promoting ROS production(Chong et al., 2017, Bhattarai et al., 2021, Wu, 2006).

Oxidative stress can be characterized by the imbalance between ROS production and the ability of cells to eliminate ROS. Research has demonstrated that oxidative stress induces apoptosis and inflammation, ultimately impacting cell survival and playing a role in the development of various diseases such as cancer, mitochondrial and neurodegenerative disorders, diabetes, chronic illnesses, and the aging process (Kudryavtseva et al., 2016, Newsholme et al., 2007, Kirkinezos and Moraes, 2001, Cui et al., 2012) Because of its heightened metabolic function and the production of ROS during the synthesis of THs, as mentioned in Section 1.3.2, the thyroid gland is especially sensitive to oxidative stress (Akinci et al., 2008, Faam et al., 2021).

The dual role of H2O2 in thyroid function

Hydrogen peroxide (H_2O_2) serves as a key factor in TH synthesis within the thyroid follicle cells. This ROS is essential for the iodination of thyroglobulin, a process catalysed by thyroid peroxidase (TPO) on the apical membrane of these cells as shown in Figure 1.6 (Ekholm, 1981). Despite its crucial role, excess H_2O_2 can be detrimental, leading to cellular damage and dysfunction.

The NADPH oxidase family members, notably DUOX1 and DUOX2 (previously known as Thox1 and Thox2, respectively, in the 9th edition of the thyroid textbook), are responsible for H2O2 generation within thyroid cells, acting in a calcium-dependent manner(Braverman and Cooper, 2013, Carvalho and Dupuy, 2013). These enzymes, along with their activators, DUOXA1 and DUOXA2, are essential for proper thyroid function and are differentially expressed in various tissues, including the thyroid gland and salivary ducts (Krause, 2004, El Hassani et al., 2005). Both DUOXs are expressed in thyroid cells, with DUOX2 being expressed at 5-times more than DUOX1 (Pachucki et al., 2004, Ameziane-El-Hassani et al., 2005). The regulation of DUOX activity is intricately linked to thyroid function; high iodine levels can suppress DUOX2-mediated H2O2 production, thereby reducing activity and iodine incorporation into the TG (Cardoso et al., 2001). This feedback system is required to prevent the Wolff-Chaikoff effect (Corvilain et al., 1988) .

Mutations in DUOX2 and its interaction with DUOXA can lead to thyroid dysfunction. For example, certain DUOX2 mutations can prevent proper enzyme transport to the cell membrane, resulting in congenital hypothyroidism(Grasberger et al., 2007). This underlines the critical balance between adequate and excessive H2O2 production in maintaining thyroid health, and emphasises the complexity of thyroid cell regulation (Rigutto et al., 2009, Grasberger, 2010).

1.3.7 Pathophysiology of Thyroid Disorders

Common thyroid disorders: hypothyroidism, hyperthyroidism, thyroiditis.

Hypothyroidism

The most common disorder linked to thyroid function is hypothyroidism. This thyroid disorder is caused by a reduction in the thyroid's ability to produce and secrete T4 and T3 hormones. TSH secretion is also elevated in primary hypothyroidism. There is another type of hypothyroidism, also known as central hypothyroidism, is far less frequently brought on by diminished thyroidal stimulation by TSH. Primary hypothyroidism is around a thousand times more common than central hypothyroidism based on the patient percentage (Persani, 2012).

There are many reasons that could cause hypothyroidism; for example, primary hypothyroidism is caused by the lack of thyroid gland function, thyroid dysgenesis or chronic autoimmune thyroiditis like Hashimoto thyroiditis and so on; insufficient thyroid stimulating hormone secretion or pituitary dysfunction is classified as central hypothyroidism or some congenital disorders like hypothalamic disease(Braverman and Cooper, 2013).

In the U.S. the latest reports indicates that among individuals aged 12 years and older, 5% of them suffer from hypothyroidism (Patil et al., 2022) In the North East of England, a retrospective examination of General Practice data (n = 66,843) revealed that the overall single-point prevalence of treated hypothyroidism was 4.5% in 2016 (Ingoe et al., 2017). According to a meta-analysis of 17 European countries the rate of hypothyroidism overall (diagnosed and undiagnosed) was determined to be 3% of the test population. Undiagnosed hypothyroidism was present in 4.9% of people (6.4% of females and 3.4% of men). Overt hypothyroidism was present in 0.8% of females and 0.3% of males, whereas subclinical hypothyroidism was present in 5.9% of females and 3.4% of men (Garmendia Madariaga et al., 2014)

Apart from the chronic autoimmune thyroiditis or congenital defects of thyroid, iodine deficiency remain the most common reason for primary hypothyroidism. In iodine sufficient areas, the main reason for the occurance

of primary hypothyroidism is auto-immune diseases like Hashimoto thyroiditis (Garber et al., 2012). In addition, it may be challenging to diagnose hypothyroidism because the initial symptoms, such as fatigue, memory loss, or weight gain, and can be difficult to distinguish from other physical illnesses in fast-paced lifestyles. Moreover, if the patient discovers the issue, it may take longer for them to recover, or their illness may worsen. For older patients typically exhibit less obvious hypothyroidism symptoms (Bensenor et al., 2012). Finally, when hypothyroidism becomes moderately or severe, it could also be accompanied by damaging other parts of the body, like low blood sodium levels or acute kidney injury (AKI)(Klubo-Gwiezdzinska and Wartofsky, 2012)

Hyperthyroidism

Hyperthyroidism, which is characterised by the overproduction of thyroid hormones (TH), is often caused by autoantibodies that stimulate the TSH receptor, specifically in Graves' disease. However, it can also be caused by the thyroid tissue that functions autonomously.

Graves' disease is the most common cause of hyperthyroidism. Thyroiditis, toxic multinodular goitre, toxic adenomas, and adverse drug reactions are additional prevalent causes. Increased thyroid hormone synthesis, excessive thyroid hormone release, endogenous or exogenous extrathyroidal sources, or a combination of these factors can all result in hyperthyroidism. The overactive thyroid results in an abnormal concentration of thyroid hormones in tissues (Kravets, 2016).

Apart from existing causes, there is an increasing trend indicating that excess iodine intake may trigger hyperthyroidism and lead to over production of THs. Over the past few decades, significant progress has been made in the global effort to avoid IDDs by iodine supplementation, such as universal salt iodization. However, due to broad environmental iodine exposure and poor monitoring, or even because of the quality control of the iodized salt, some regions of the world are reporting problems linked to iodine excess.

Iodine status of 78,470 participants in a cross-sectional nationwide study in China indicates the median UIC level are 177.89 μg/L (Yongze Li, 2020). In

addition, the authors found that the rate of thyroid disorder among all the participantswas 0.78% for overt hyperthyroidism, 0.44% for subclinical hyperthyroidism, 0.53% for Graves' disease, 1.02% for overt hypothyroidism, 12.93% of subclinical hypothyroidism. Li's result indicates that as a nation with vast territory, even though the problem with hyperthyroidism existed, from a nationwide point of view, enhancing the popularization of iodized salt is a much more urgent problem.

In contrast, with the iodized salt introduced in Denmark (0.12%)(Bülow Pedersen et al., 2006) , Zimbabwe and Democratic Republic of Congo (F. DELANGE, 1999) all reported the increase of the cases of hyperthyroidism. However, no major studies on the detrimental impact of elevated iodine intakes have yet to be conducted.

1.4 Aim and Objectives

The overarching aim of this project was to:

1. To investigate the iodine status of the UK population and elucidate the implications of dietary iodine fluctuation.
2. To explore the iodine fluctuation and investigate the changes in thyroid hormone production pathway using in vitro model.
3. To examine the cellular stress responses induced by reactive oxygen species (ROS) in relation to iodine fluctuations.

The objective of this study was to

I. To investigate iodine levels in the UK population and understand the main contributor of dietary iodine intake using NDNS. Investigate the main contributor of iodine fluctuation in iodine-rich food sources in the UK.

II. To develop an in vitro cellular model to simulate fluctuating iodine levels using a thyroid cell line.

III. Utilizing the iodine fluctuation model investigate the effects on the production pathway of thyroid hormones.

IV. Evaluate the effects of iodine fluctuation on cellular stress by examining reactive oxygen species (ROS) levels induced by hydrogen peroxide (H2O2) in thyroid cells.

V. Investigate the protective effects of triiodothyronine (T3) on various cell types against ROS-induced cellular stress resulting from fluctuating iodine levels.

Chapter 2 General Method

2.1 Cell culture

All cell culture work was conducted in Class II biological safety cabinets (Model AC2-4S1, Esco Micro Pte). All cell types used and described in this section were cultured in a humidified incubator (Galaxy S+, RS Biotech) at 37℃ 5% CO_2.

Prior to any cell culture work, non-sterile equipment was autoclaved at 121℃ for 15 minutes (MP24 Control System, Rodwell Scientific Instruments) and dried in the cabinet before use. All cell culture work was carried out using sterile techniques.

2.1.1Used Cell lines

2.1.1.1 HepG2

Immortalized human liver cancer cell line **HepG2** was originally isolated from a 15-year-old Caucasian male that have differentiated liver carcinoma (Donato et al., 2015). HepG2 was cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM, D6546, containing 4500mg/L glucose and sodium pyruvate without L-glutamine, Sigma-Aldrich) supplemented with 1% (v/v) L-glutamine (25030-024, Gibco, Life Technologies Ltd), 1% (v/v) penicillin-streptomycin (100µg/ml, Penstrep, 11548876, Gibco, Life Technologies Ltd) and 10% (v/v) fetal bovine serum (FBS, 10500-064, Gibco, Life Technologies Ltd).

The growth medium containing all supplements was sterile filtered using 0.2µm syringe filters (250ML/431096 or 500ML/431097, Corning Incorporated) before use. The HepG2 cells were incubated with the growth medium in a T75 flask and continued to grow till they reached around 70-80% confluence. In addition, the culture medium was changed every two days with the purpose of keeping the cells in the best possible proliferative state.

The HepG2 cells were kept between passages number of 35 to 50 for experiments and used before passage number 50.

2.1.1.2 HK2

Human Kidney 2, also known as HK2, is an immortalized human proximal tubular epithelial cell line that is derived from normal human kidney tissue. Originally HK2 cells were collected from male adult human kidney cortex followed by incubation with a recombinant retrovirus that contains human papillomavirus (HPV16) E6/E7 genes (Ryan et al., 1994). The HPV E6/E7 genes were shown to immortalize epithelial cells without changing their functions. HK2 was cultured in the same condition with HepG2 cells (DMEM-D6546 supplemented with L-glutamine, a mix of penicillin, streptomycin and 10% FBS). The HK2 cells were used before passage number 30.

The culture media was changed every two days and the cell was passaged to new flask after it reach about 80% confluence. The cell culture procedures, including splitting, culturing, freezing, thawing and counting, for HK2 were identical to all other cell lines.

2.1.1.3 HEK293

Human embryonic kidney 293 cells (referred to as **HEK293**), were originally isolated from a healthy legally aborted human fetus and then transfected with sheared adenovirus types 5 DNA (AD5) by Graham (1977). Due to the transfection of the adenoviral genes, resulting in the HEK cell can work very efficient at producing high amounts of recombinant proteins.

The cell culture procedures and the culturing environment for HEK293 were identical to the HepG2 and HK2 cell line.

2.1.1.4 C2C12

Immortalized mouse myoblast cell (**C2C12**) was a sub clone cell line of the mouse myoblast cell line established by D. Yaffe and O. Saxel. It was widely used for its characteristics of differentiating rapidly, forming contractile myotubes and producing muscle proteins (McMahon et al., 1994).

The culturing procedures for C2C12 cells were similar to HepG2 and HK2 cell line apart from the different medium was used in culturing C2C12 (DMEM-D5796, Sigma-Aldrich) which was supplemented with 1% (v/v) L-glutamine, 1% (v/v) of penicillin/streptomycin and 10% (v/v) FBS.

The C2C12 cells were used before passage number 30 in this project, and it was passaged to new flask after it reach about 80% confluence; the culture media was changed every two days.

2.1.1.5 NThy-ori 3-1

Nthy-ori 3-1 cell line is an immortalised thyroid follicular epithelial cell which was obtained from the thyroid of a normal female. The obtained primary thyroid cells were transfected with a plasmid containing an origin-defective SV40 genome (SV-ori) that was able to immortalise them while retaining functions as it can be used in the study of human thyroid growth and mRNA expression profiling, etc(Lemoine et al., 1989, Kim et al., 2017).

The Nthy-ori 3-1 cells were grown with RPMI-1640 medium (Gibco) supplemented with 1% (v/v) of penicillin-streptomycin and 10% (v/v) FBS. The culture media was changed every two days and the cell was passaged to new flask after it reach about 80% confluence or once a week.

2.1.2 For splitting cells

All target cells were split into new flasks after reaching 80% confluence, so that the cells would be able to continue growth in an optimal proliferative state. When the cells reached confluence for splitting, the culture medium was removed, before the cells were washed with pre-warmed sterile PBS solution, and then 2 ml of trypsin-EDTA solution (0.25% trypsin-EDTA, Gibco Life Technologies) for 5 min in a 5% CO2 humidified incubator. Next, 8 ml of growth medium was added to terminate the trypsin activity. The trypsin and medium mixed solution were then collected from the flask before being transferred to a 20 ml universal flask and centrifuged at 1,000 rpm for 5 min in a centrifuge (5702R Eppendorf UK, Ltd.). The supernatant was disposed, and the cell pellet was resuspended in 10 ml of growth medium and 1 ml of cell suspension was diluted to a new T75 flask with 13 ml fresh growth medium.

2.1.3 Culturing cells

Cells stored in CryoPure tube in liquid nitrogen were thawed at 37℃ beads bath until it is around 90% thawed, then it was transferred inside the biosafety hood and pipetted into a T75 cell culture flask. Followed by adding 13ml of prewarmed cell growth media and checking cell condition under a microscope. Afterwards, the flask was moved to a 37℃ humidified incubator with 5% CO2. The medium was changed after 24h of the first-day incubation and then changed every 48 hours.

2.1.4 Freezing and thawing cells

In the project, different cell types were kept in between different passage numbers, which are described in their own section, to avoid alteration in gene expression and maintain sufficient cell stock for the following experiment. In addition, to maintain a sufficiently low passage number in the cell stock, cells were split and stored frozen in liquid nitrogen at a low passage number for future use.

After the low passage number of cells cultured in the T75 flask reached 80-90% confluence, it was dislodged using 2 ml of the trypsin-EDTA solution and centrifuged to obtain the cell pellet described in 2.1.2 section. The medium in the cell pellet was then removed, leaving the cell pellet resuspended in 10% DMSO (dimethyl sulfoxide, D/4120/PB08, Fisher Chemicals) in an FBS mixture solution. The resuspended solution was then pipetted into well-labelled CryoPure tubes (72.379, Sarstedt), containing 1 ml per vial, before placing all the tubes in an Mr. FrostyTM Freezing Container (5100-0001, Thermo Scientific TM). The Mr Frosty Container contained 100% isopropanol (P/7500, Fisher Scientific); in order to maintain better conditions for freezing cells, the isopropanol was changed every five freezing procedure times. The container with cell stock tubes was then labelled and stored in a -80 °C freezer overnight for the cells to gradually freeze before moving the CryoPure tubes into liquid nitrogen for long-term storage.

To thaw frozen cells from liquid nitrogen, we warmed the tube in a heating bead bath at 37 °C and followed the culture procedures described in section 2.1.3.

2.1.5 Cell counting

Prior to seeding cells onto various types of culture plate, they were counted in order to make sure a consistent number of cells was seeded into each well.

This is important for reducing experimental variability. The Hawksley haemocytometer (BS748-AC1000) was used to count the cells after resuspension in fresh media (see 2.1.2 splitting cells section). 10ul of well-mixed cell-media suspension was pipetted in between the chamber of the haemocytometer and the cover glass and the average cell number of the three or four main chamber squares was counted and calculated to calculate the total cell number which was based on the following formula:

Total Cell number = *Average cell number x 10^4 x Total volume of cell suspension (ul)*

In addition, the number of cells needed for different sizes of well according to the plate type was calculated based on the following formula, which determined the volume of the stock cell suspension required to reach the required cell number.

Required Volume (µl) = *(cell number needed/ Total cell number) x Total volume of cell suspension (µl)*

The cell number needed for each plate type and the media volume for each plate type was shown in the table 2.1.

Plate types	Product number	Growth media volume (ml)	Cell number per well
6-well plates	3516, Corning Costar	2	0.3×10^6
12-well plates	3513, Corning Costar	1	0.1×10^6
96-well plates	3596, Corning Costar	0.2	0.05×10^6
	Blackwalled code*	0.2	0.05×10^6

Table 2. 1 **Seeding density of different plate types in cell culture**

(According to Thermo fisher scientific)

2.2 Cell viability

2.2.1 MTT

Cells were grown in 96 well plates until they reached 80% confluence, then incubated with hormone-free media supplemented with L-glutamine, pen-streptomycin (100µg/ml each) and 10% charcoal-stripped FBS (A33821, Gibco, Life Technologies Ltd) for 24 hours. Afterwards, the treatment medium was applied to the cells for 24 hours or 48 hours accordingly.

Following treatments, the medium was removed and 100ul of HBSS was added to each well. Then 10µl of 12mM 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, V13154, Molecular Probes, Life Technologies) was added to each well before incubating the plate at a 37℃

humidified incubator with 5% CO2. Following 4 hours of incubation, 85ul of mixed solution in the well was removed, then 50ul of DMSO was added in each well, pipetted up and down for a few times before moving the plate into the same incubator for 10 minutes incubation.

Afterwards, the plate was read using a plate reader (Model 680 XR microplate reader, Bio-Rad) at 550nm absorbance. An equal volume of plain HBSS with DMSO was used as a negative control to the sample. Each treatment group had at least triplicates in the same plate, and the same treatment plate was repeated on two other plates at the same time as well in order to generate a minimum of 3 experimental replicates.

2.2.2 CCK8 ASSAY

Cells were seeded into 96 well plates to conduct the CCK assay and grown overnight before treatment. Following the treatment, 10μl of CCK-8 kit (96992, Sigma-Aldrich) was added to each well or, depending on the volume in each well, added 10% (v/v) of the media volume of the CCK-8 solution. Afterwards, the plate was incubated for 4 hours in a 37℃ humidified incubator with 5% CO2. Following the 4 hours of incubation, the plate was ready for reading at 450nm absorbance using a plate reader (Model 680 XR microplate reader, Bio-Rad). Each treatment group was measured in triplicate in the same plate and again each treatment plate was repeated to generate a minimum of 3 experimental replicates.

In the MTT assay, the MTT is reduced to purple-coloured formazan, which is dissolvable in DMSO, in the mitochondria of living cells (Figure 2.1). CCK-8, on the other hand, was using WSTs (water-soluble tetrazolium salts), WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) to be exact. It can be reduced by cellular dehydrogenases to generate an orange-coloured water-soluble form of formazan (Figure 2.2). Additionally, it has higher detection sensitivity and lower cytotoxicity than MTT (From CCK-8 kit product description, 2018).

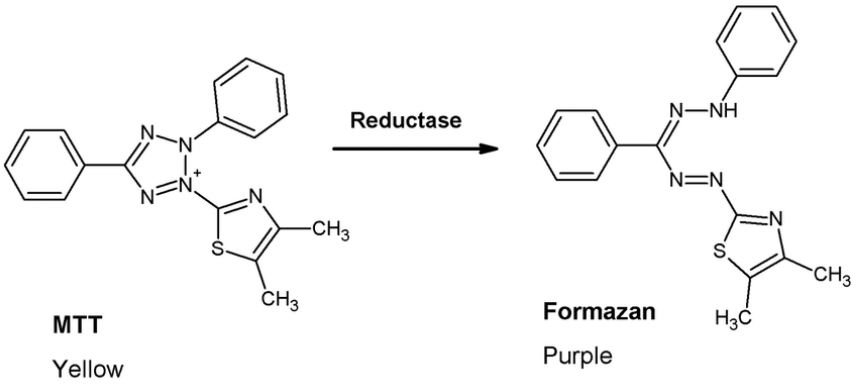


Figure 2. 1 MTT converted to formazan

Figure is taken from Wolfgang E Trommer (2012) The ribosome-inactivating protein gelonin and parts thereof to be employed for a potential treatment of cancer.

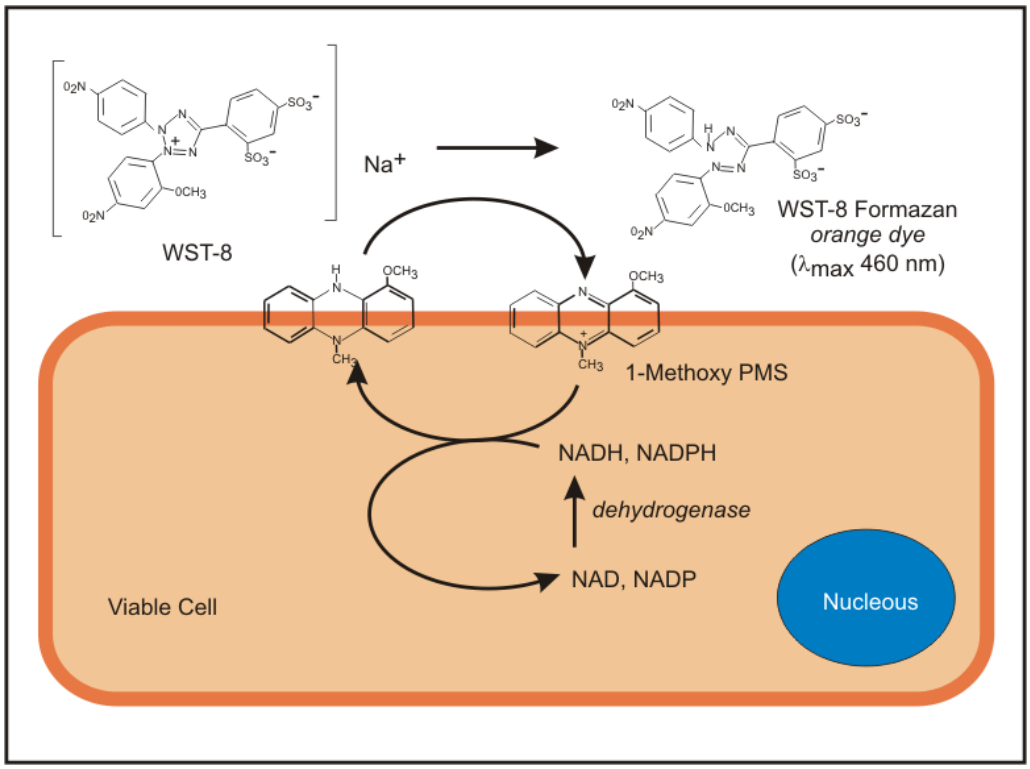


Figure 2. 2 WST-8 Assay Mechanism

Figure is taken from Paul Held (2009) An Absorbance-based Cytotoxicity Assay using High Absorptivity, Water-soluble Tetrazolium Salts.

The results of both MTT assay and CCK-8 assay were then presented as cell viability, it was calculated as the formula presented below:

Cell Viability = ((absorbance of treated group – absorbance of negative control group) / (absorbance of control group – absorbance of negative control group)) x 100%

2.3 Reactive Oxygen Species (ROS) Measurement

The reactive oxygen species (ROS) was measured via the fluorescent dye-based kit CM-H2DCFDA (C6827, Introgen, Life technologies). H2DCFDA (2′,7′-Dichlorodihydrofluorescein diacetate) is a fluorogenic reagent that is able to detect reactive oxygen intermediates in cells. The CM-H2DCFDA is one of the chloromethyl derivatives of H2DCFDA since it can be preserved longer than H2DCFDA in live cells, which is a better indicator for ROS in cells.

Cells were seeded in black-walled clear-bottom 96 well plates 24 hours before any treatment. Then, the treatment was added to the cells for different length of time based on different experiments, which will be explained in the specific chapter. Each vial of CM-H2DCFDA needs to add 8.6µl of DMSO to get the stock solution at 10mM, which the stock solution would be diluted in HBSS up to 5uM as the working solution; all working solution was made fresh upon use.

The treatment media in the 96 well plate was removed and replaced with the CM-H2DCFDA working solution, the plate was then put back into the 37℃ humidified 5% CO2 incubator for a certain period of time according to different experiment requirements before the absorbance read.

The fluorescent absorbance was read using the FLUOstar Optima microplate reader at the excitation wavelength of 485nm and the emission wavelength of 520nm. Each treatment was triplicated in each plate, the treatment itself was also repeated in the other two plates as well.

2.4 Enzyme-linked immunosorbent assay (ELISA)

2.4.1 Plate Preparation

Human Thyroxine ELISA Kit (fT4) (ab108662) was used, the kit comes with a 96-well plate that is supplied and ready for use. There is no need to rinse the plate before using it or adding reagents. Unused wells can be removed as rows and taken from the holder but must be placed back into the plate packet and kept at 4°C.

2.4.2 Assay Procedure

Prior to use, brought all reagents, samples, and controls to room temperature. 50X Washing Solution should be diluted with deionized water to create 1X Washing Solution. Mix thoroughly and gently; the diluted solution is stable for 30 days at 2-8°C, but we made them freshly in every experiment.

Add 25 µL standard, control or sample into their respective wells. Add 100 µL Thyroxine (T4)-HRP Conjugate to each well. Leave a blank well for substrate blank. Cut the foil supplied in the kit to how much you need, and cover wells with the foil. Then incubate the plate for 1 hour at room temperature. After the incubation period is over, peel off the foil, aspirate the contents of the wells, and wash them three times in 300µL of wash buffer. Be careful not to allow the reaction wells to overflow during this process.

After each washing cycle, rotate the plate over and gently tap it on a piece of absorbent paper towel to remove any excess solution. Then add 100 µL TMB Substrate Solution into all wells. Reuse the foil and incubate the plate for 15 minutes at room temperature in the dark.

After the incubation, add 100 µL Stop Solution into all wells and tap the microplate gently with your hand from the side. Measure the absorbance of the sample at 450 nm within 30 minutes.

2.5 Real-Time Quantitative PCR

2.5.1 Sample collection for RNA extraction

The cells were seeded in 6 well plates (3516, Corning) and incubated until they reached around 70% of confluence before being treated according to the protocol designed. After treatment, the treatment media was completely discarded before washing the cell with ice-cold PBS twice. Then, 300µl of ice-cold RNase-free PBS was added to each plate well before using a sterile cell scraper (25cm 83.1830, Sarstedt) to dislodge all the attached cells and pipette the mix of cells in PBS into well labelled 1.5ml Eppendorf. Subsequently, the cell was spun down to a cell pellet at 0℃, after discarding the supernatant, it was then resuspended with 300µl ice cold PBS. Later, all samples were stored at -80℃ before processing.

2.5.2 RNA extraction

In order to extract total RNA from cells, Roche high pure isolation kit (#11828665001, Roche) was used, and all procedures were conducted following manufacture's protocol in a MACH-AIRE fume hood (K-CT1100, recirculating fume hoods, Monmouth Scientific).

Samples were put on ice, and 400µl Lysis-Binding buffer was added after defrosting; then, all samples were centrifuged in the microcentrifuge for 1 minute at 13,000 rpm 4℃. After the centrifuge, the sample mixture was transferred to a well-labelled high filter tube combined within a collection tube before spinning down at 13,000 rpm 4℃ for 1 minute. Then the flowthrough liquid in the collection tube was discarded.

90µl of DNase incubation buffer and 10µl of DNase I per sample were prepared and mixed before use. The mix was then pipetted into the filter tube, and the incubation was conducted at room temperature for 15 minutes.

After the incubation, 500µl of washing buffer I was added to the filter tube and centrifuged at 13,000 rpm for 1 minute. The fluid in the collection tube was discarded afterwards. The sample was then washed with 500µl of wash buffer

II before centrifuging at 13,000 rpm for 1 minute as well. The fluid in the collection tube was discarded again. 200µl of wash buffer II was added to the filter tube again and spun down at 13,000 rpm for 2 minutes, this time to remove the remaining wash buffer in the filter tube. The fluid in the collection tube and the old collection tube were both discarded; the new collection tube was applied. Finally, 50µl of RNase-free water (Elution buffer) was cautiously added to the filter tube and made sure it was directly pipetted onto the whole filter, then spun down at 13,000 rpm for 2 minutes to elute the extracted RNA.

The RNA samples were kept on ice, ready for testing the concentration or stored at -80℃ for later assessment.

2.5.3 Quantify total RNA using Nanodrop

To assess the concentration of total RNA level extracted from the sample, Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific) was used. Before using the Nanodrop, the pedestal was cleaned with lint-free tissue. Next, 1.5µl of the RNase-free water was pipetted onto the pedestal for blanking, and then the same amount of the sample was pipetted onto the pedestal to read the absorbances. The same sample's absorbance was read at least twice to ensure consistent readings for the concentration quantity(ng/µl). The RNA sample purity could be assessed via absorbance ratio at 260 and 230 nm; the ratio between 2 to 2.2 would be considered the purer RNA sample.

All RNA samples were then diluted to 50 ng/µl concentration with RNase-free water (#W4502, Nuclease-Free Water, for Molecular Biology, Sigma-Aldrich) and stored at -80℃ until future use.

2.5.4 cDNA synthesis

The first strand of complimentary DNA (cDNA) was conducted using the RevertAid RT Kit (K1691, Thermo Fisher Scientific), following the provided protocol.

Using all equally diluted RNA samples (50 ng/µl) during this procedure, according to the table 2.4, 10µl of 50ng/µl RNA (equivalent to 500ng) was pipetted into a 200µl nuclease-free tube, then 1µl of random Hexamer primer and 1µl of RNase-free water was also added to the tube.

Then the mixture reagent was mixed by gently pipetting up and down a few times before incubating in a GeneAmp PCR system 9700 (Thermo Fisher Scientific) at 65℃ for 5 minutes. Afterwards, all the tubes were cooled on ice while making the following step mixture.

All the components shown in table 2.5 for the second step mixture was added to an RNase-free tube, making sure the amount of all components added into the tube was calculated correctly per sample. Then 8µl of the second step mixture was added into each tube kept on ice, mixed gently and centrifuged all tubes briefly.

Afterwards, all tubes were transferred back onto the GeneAmp PCR system and incubated at 25℃ for 5 minutes, followed by 1-hour incubation at 42℃; then, the reaction was terminated by heating up to 70℃ for 5 minutes and cooled down to 4℃. The stock cDNA was then made by adding 80µl RNase-free distilled water to the tube and diluting the incubated cDNA samples in a 1:5 ratio to have a final volume of 100µl. These stock cDNA samples were well labelled, kept at -20℃ for recent use or stored at -80℃ for long-term storage.

2.5.5 Primer Design

Primers were designed using Primer Express Software Version 3.0.1 (11983, Applied Biosystems) and listed in table 2.5 of the primer information of studied genes.

When designing the primers, we tried to make sure either the forward or reverse primer fell on an exon-exon boundary. The primer was run in the BLAST (Basic Local Alignment Search Tool) to make sure it only detected the gene of interest. Afterwards, the primer set was purchased from Sigma-Aldridge.

Once the designed primer arrived, it was then diluted in the appropriate amount of RNase-free water and given a 100uM stock concentration referring to the manufacturer's indication sheet. We used the primer stock solution to make the working solution at 10uM as a 1 in 10 ratio in a well labelled RNase-free 1.5ml tube and keep it in a separate place to the stock for storage.

Before the primer was used in the qPCR experiment, it tested via melt-curve analysis using Roche Lightcycler 480 and stored at -20℃.

Reagents	**Volume(µl)**
Template RNA (50ng/µl)	10
Random Hexamer Primer	1
Nuclease-free Water	1
Total volume	12

Table 2. 2 Step 1 of RevertAid RT Kit cDNA synthesis mixture reagents

Reagents	**Volume(µl)**
Reaction Buffer(5x)	4
10mM dNTP Mix (dATP, dCTP, dGDP, dTTP)	2
RiboLock RNase inhibitor(20U/µl)	1
RevertAid RT (200U/µl)	1
Total volume	8

Table 2. 3 Step 2 of RevertAid RT Kit cDNA synthesis mixture reagents

Gene Name	Forward(F) or Reverse(R)	Sequences (5' to 3')
Gadph	F	ATATGATTCCACCCATGGCA
	R	TGGAAGATGGTGATGGGATT
NIS	F	CCCCCAGCTCAGGAATGG
	R	AGGAGATGGCATAGAAGCTGTCA
TSHR	F	AATGGGGTGTTCGTCTCCAC
	R	AAGCTTCAGAGTCTGCGTACTG
TPO	F	CCGAAATCAGCAGGACGACT
	R	AAAGGCCAGACAAATGCAGC
TGF-B1	F	GTGGAAACCCACAACGAAAT
	R	CGGAGCTCTGATGTGTTGAA
PAX8	F	TTCCCTGGGCTCCACCTACT
	R	CTGATCACTGTCATCCATTTTCCT
DUOX2	F	CTCAGGACCACCATGCTATACATC
	R	GGAGTTGAAGAAGGGCTCGAA
PDS	F	GTGAACGTTCCCAAAGTGCC
	R	TTTGACAATCACCCGCAGTG

Table 2. 4 RT PCR primers for human gene measured

2.5.6 Real time quantitative PCR (RT PCR)

The RTqPCR was conducted using the Roche Lightcycler 480 (Roche Diagnostics), this method measures the relative mRNA transcript abundance of target genes in tested sample. As described in section 4.5 all the primer for the gene of interest was designed and tested before use in the RTqPCR experiment. After the first cDNA strand is generated, primers designed specifically for each gene will amplify the target sequence of the cDNA, resulting in a double-stranded DNA product.

RT PCR was performed on Roche Lightcycler 480 in order to measure the transcript abundance of the gene of interest. The LightCycler 480 SYBR

Green I master (04887352001, Roche Diagnostics) was used in the mixture to interpose between double-stranded DNA to generate a fluorescent signal for the instrument to pick up the measurement for further quantification.

The 384 well optical plates were used to conduct the PCR experiment; 5µl of cDNA sample was loaded to each well along with 10µl of the master mix (listed in table 2.5) of all the reaction reagents required. Triplicates of the same cDNA sample were loaded into the same 384 well plates and triplicates of the negative control with RNase-free water with the master mix.

After finishing loading the plate, the optical sealing film was used to seal the well, preventing cross-contamination and sample evapouration. Then the sealed plate was put into the plate spinner (MPS C1000, Mini PCR Plate Spinner Centrifuge, Labnet International) and centrifuged for a few seconds before inserting the plate into the instrument. The protocol for the SYBR Green was described in Table 2.6.

The cross point (CP) value indicates the cycles of the fluorescent signal could be detected when it crosses the threshold. CP value could be used to calculate the concentration of the targeted PCR product; the lower the cp value indicates, the higher level of the transcribed PCR product of the target gene.

	Volume (µL)
SYBR Green I Master mix	7.5
Forward Primer	0.45
Reverse Primer	0.45
RNase-free water	1.6
Total volume per well	**10**

Table 2. 5 Volumes of the reaction reagent used in RTqPCR in each reaction well.

		Temperature (℃)	Time	Cycle number
Pre-incubation		95	5mins	1
Amplification	Denaturation	95	10sec	45
	Annealing	60	15sec	
	Extension	72	15sec	
Melt curve		95	5sec	1
		65	1min	
		97		
Cooling		40	10sec	1

Table 2. 6 RT qPCR SYBR Green program template used for Roche LightCycler 480.

2.5.7 Quantification of the transcript abundance using standard curve

The quantitative PCR (qPCR) sample quantification was performed under the guidance of our experienced Dr. Zoe Daniels and Guescini et al. (2008). Crossing point (CP) values were obtained for all individual cDNA samples, and normalised to a control gene (e.g., GAPDH). The samples were evaluated to ensure that their CP values were within two standard deviations (SD) of the mean CP for all samples. Those with CP values falling outside this range were excluded from the cDNA pool for the standard curve construction.

Aliquots from each qualified sample were combined to construct an accurate cDNA standard pool. This pool was then used to prepare serial dilutions at ratios of 1 (undiluted), 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128 to cover the appropriate CP range. The most concentrated sample in these dilutions was assigned a concentration code of '1' on the standard curve.

A standard curve was generated by running each dilution in triplicate on a qPCR plate. Analysis of the standard curve and subsequent CP values for unknown samples was conducted using the LightCycler software, which

facilitated the determination of target gene concentrations. In addition, a standard curve can provide amplification efficiency, test primer efficiency, and serial dilution. Moreover, to test whether the primers worked properly, melt curve analysis was performed to check the specificity of the primers.

2.6 Western Blot

2.6.1 Preparation of protein reagents

In order to quantify the abundance of the protein of interest, the analysis of western blot was conducted. To prepare for the reagents for sample collection, the stock of 2X SDS (2X Sodium Dodecyl Sulfate) buffer was prepared as shown in Table 2.4, then dilute the 2X SDS into 1X SDS (5ml of 2X SDS with 5ml of Elix water) as the **lysis buffer** for sample collection. Added two of the tablet of protease inhibitor cocktail (complete, 04693124001, Roche) in 10mL of the lysis buffer. Both 2X SDS stock and 1X SDS were stored at -20℃.

Reagents	Volume(mL)	Product code
Glycerol (≥99%)	2	G5516, Sigma-Aldrich
1M Tris-HCL (pH 6.8)	1.25	NA
SDS Solution (10% w/v)	4	NA
1M DTT (0.154g/mL)	1	D9779, Sigma-Aldrich
Bromophenol Blue	Few grains	53H3640, Sigma-Aldrich
Elix Water	1.75	NA
Total Volume	**10**	

Table 2. 7 The components in 10mL 2X SDS

2.6.2 Western blot sample collection

The cells were seeded in 6-well plates and treated accordingly. Each treatment group was replicated across three different plates (n = 3 for each group). Following treatment, the medium was carefully aspirated from each well, and the cells were washed with 1 mL of ice-cold phosphate-buffered saline (PBS). Subsequently, 300 µL of lysis buffer was added to each well. The cells were then scraped and transferred into labeled 1.5 mL Eppendorf tubes. To preserve the sample integrity, all samples were maintained on ice throughout the collection process.

2.6.3 Quantification of protein concentration using 2D Quant

The protein concentration of collected samples was quantified using 2D quant kit (80-0483-56, GE Healthcare Life Sciences) prior to being used for western blot analysis. The protocol of 2D quant kit was performed according to the manufacturer's protocol. Before conducting the 2D quant analysis, all samples were heated to 95-100℃ for 5 minutes to denature the protein. Samples were centrifuged at 13,000rpm for 5 minutes using Microfuge 22R microcentrifuge (Beckham Coulter) and afterwards placed on ice till further step.

A standard curve was applied using supplied stock of 2mg/ml Bovine serum albumin (BSA), via adding the amount of 0µl, 5µl,10µl,15µl, 20µl and 25µl of the supplied BSA to separated 2ml microcentrifuge tubes, in order to generate the final protein concentration as 0ug, 10ug, 20ug, 30ug, 40ug and 50ug.

Both the standard curve and all the sample was repeated two times at least.

10µl of the sample was pipetted into 1.5ml microcentrifuge tube and labelled cautiously, then added 500µl Precipitant Solution to the standard curve and all the samples. All the samples, including the standard curve, were then vortexed briefly and incubated at room temperature for 2-3 minutes. Thereafter, 500µl of the Co-precipitant Solution was added to all tubes and mixed briefly, then all samples were centrifuged at 13,000rpm and 0℃ for 10

minutes. The protein pellet down in the tube, carefully remove the supernatant from each tube. Afterwards, all the samples were centrifuged again to remove any remaining supernatant and make sure the pellet was preserved. 100µl of the Copper Solution and 400µl of Elix water was added to each tube and vortexed until the protein pellet within the tube was completely dissolved. The working colour reagent was freshly mixed before adding into each tube, and it was mixed by 100 units of Colour Reagent A with one unit of Colour Reagent B (e.g.100 µL Reagent A with 1 µL reagent B). Each sample was added with 1ml of working colour reagent and mixed well by inversion before incubating at room temperature for 20 minutes.

After the incubation, all samples were transferred to individual cuvette (10x4x45mm, 67,742, Sarstedt) for reading the absorbance at 480nm on a UV/Vis Spectrophotometer (Ultrospec III, Pharmacia LKB), the absorbance was read within 40 minutes of the colour reagent being added to the sample. Again, Elix water was used as the reference.

The standard curve was generated for protein quantity related to absorbance values and used Microsoft Excel to generate the equation; it then is used to quantify unknown sample protein concentration.

All samples were normalized to 1.2 ug/µl or 2ug/µl with 1X SDS loading buffer after the quantification and stored at -20℃ for short-term use or -80℃ for long-term storage.

2.6.4 Polyacrylamide Gel Electrophoresis (PAGE)

Prior to the Western Blot analysis, samples were thawed on ice. 10x Tris-glycine-SDS PAGE buffer (EC-870, National Diagnositics) was diluted 1 in 10 with Elix water for using as 1x running buffer. The 4-15% Criterion TGX Precast Midi Protein Gel (18-well: 5671084; 26-well: 5671085, BIO-RAD) was slotted in a gel tank and made sure it was fully submerged in 1x running buffer within the tank. All normalized samples were loaded into the Precast Protein Gel with equivalent amount of 15ug of protein, as well as a 10µl of SDS

stained protein standard (Color Prestained Protein Standard, Borad Range(10-250kDa), P7719S, New England BioLabs) on the same gel.

The gel was then run on a Bio-Rad Criterion Cell unit and connect to a PowerPac (PowerPac^{TM} Basic Power Supply, Bio-Rad) that set up at 200V for 40-45 minutes until the smallest protein marker run off the bottom of the gel.

The transfer buffer (Table 2.7) was made freshly and mixed carefully with a magnetic flea on a magnetic stirrer plate (Stirrer SB 161-3, Stuart), three sponges and four pieces of sponge-sized filter paper were soaked in the transfer buffer 20 minutes prior to the transfer step. The nitrocellulose membrane (Amersham Protran Supported 0.45NC, 10600018, Amersham, GE Healthcare) was cut to appropriate size presoaked in Elix water for 10 minutes then soaked in transfer buffer before use. After the polyacrylamide gel electrophoresis had stopped, the gel was cautiously removed from the plastic cassette, the top left corner of the gel was cut to help orientate in the transfer cassette. It was kept in the transfer buffer before being moved into the transfer cassette to prevent it from drying out. A transfer unit was set up in the exact order: from the black side of the cassette, two sponges, two pieces of filter paper, PAGE gel, nitrocellulose membrane, two pieces of filter paper and one sponge at the clear side of the cassette.

After each layer of the materials was added to the transfer cassette, a glass rod was used to roll out all the air bubbles to prevent the bubble obstacle in the transfer procedure. Then the transfer cassette was tightly clipped together to further secure no air bubbles interference during transfer.

Before positioning the transfer cassette in the tank, a magnetic stir bar was placed at the bottom of the transfer tank (Trans-blot cell No.36S/3076, Bio-Rad). The gel side (black cassette side) was positioned closer to the negative electrode, and the nitrocellulose membrane side (clear cassette side) was positioned close to the positive electrode, which allowed the protein to migrate from the negative to the positive side and bind with the membrane during the transfer. Two cooling packs were placed in the transfer tank to act as the cooling system to prevent overheating during the transfer process. The

transfer tank was filled up with transfer buffer and then put on the magnetic stirrer plate to keep the magnetic stir spinning during the transfer. The electric current from connected to the PowerPac for the transfer was set up as 0.5A, and the process was 2 hours.

Product name	Quantity	Brand and Product code
Glycine	60g	G/P460/53, Thermo Fisher Scientific
Tris Base	6g	BP152-1, Thermo Fisher Scientific
Isopropanol	100ml	P7500/PB17, Thermo Fisher Scientific
Elix Water	Fill up to 2L	NA

Table 2. 8 Quantity of components used to make transfer buffer.

After the transfer procedure was finished, the transfer cassette was taken out of the tank, and the nitrocellulose membrane was cut to an appropriate size related to the gel. The membrane was then moved into a container, and Ponceau-S was applied to the membrane to ensure the transfer was successful. The Ponceau stained membrane was then rinsed with Elix water to remove excess Ponceau-S from the membrane.

1x TBS-T was made using 10xTBS (described in table 2.9) prior to the experiment, with 1L of 1x TBS-T solution, 100ml of 10xTBS used with 1ml of the Tween-20(P1379, Sigma Aldrich) and filled up with Elix water. 1x TBS-T working solution was thoroughly mixed with a magnetic flea within the bottle and on the magnetic stirrer plate.

The nitrocellulose membrane was then washed with 1x TBS-T on a see-saw rocker (SSL4, Stuart; washing speed: 28 oscillations/min) for 10 minutes to completely destain the membrane.

It is necessary to block the membrane before applying primary and secondary antibodies to the membrane to prevent the antibodies bonds to non-specific background on the membrane. The membrane was blocked with 5% Marvel instant dried skimmed milk (Premier Brands UK) in 1x TBS-T for 1 hour at room temperature on the see-saw rocker (speed: 24 oscillations/min).

Product name	Quantity	Brand and Product code
Sodium Chloride	90g	BPE358, Fisher Scientific
Tris (1M, PH 7.5)	200ml	BP152-1, Fisher Scientific
Elix Water	Top up to 1L	

Table 2. 9 Quantity of components for 10x TBS

2.6.5 Incubate with primary and secondary antibodies and Detection

The primary antibody was diluted freshly with 5% powdered milk (Marvel) in 1x TBS-T, and the dilution factor was according to the protocol suggested by the manufacturer. After blocking, the membrane was incubated with primary antibody at 4℃ overnight on a see-saw rocker. The next day, the primary antibody was removed and replaced with 1x TBS-T for washing on the rocker; 1x TBS-T was changed every ten minutes and repeat the washing step at least six times. The membrane was then incubated with the secondary antibody with the required species at a 1:10000 ratio in 5%milk in 1x TBS-T for 1 hour at room temperature on the see-saw rocker. Afterwards, the membrane was washed with 1x TBS-T for 10 minutes, repeated six times.

After the wash process, the nitrocellulose membrane was then ready for detection. ECL western blotting detection reagent (RPN2209, GE Healthare) or ECL select western blotting detection reagent (RPN2235, Cytiva) was used to detect the protein band. The luminol solution and peroxide solution were freshly mixed with a 1:1 ratio prior to use; the total amount of the mixed solution needed was determined due to membrane size. The membrane was taken out from 1x TBS-T, dipped dry on a filter paper before placed on a plastic sheeting on a flat surface. Mixed detection solution was pipetted onto the membrane evenly and incubated for 5 minutes according to the manufacturer's protocol. After incubation, the excess solution was removed, the membrane was put in a transparent plastic wrap, and then it was placed in the Chemidoc MP Imaging System (Bio-Rad) to make sure the protein side was up and scanned for bands.

The same membrane was then stripped with stripping buffer (Table 2.10) and re-probed again with the primary antibody as described above. Use hotplate to reheat the stripping buffer back to transparent condition again if the stripping buffer gets cloudy due to storage conditions.

The membrane was stripped by using at least 30ml of the stripping buffer incubated at room temperature on the see-saw rocker for 10 minutes. Afterwards, the membrane was washed with PBS for 10 minutes and repeated twice; then, the membrane was washed with 1x TBS-T for another 10 minutes and repeated twice. Then the membrane was ready to block again with 5% milk in 1x TBS-T for 1 hour. To confirm that the original antibody on the membrane was successfully stripped off, incubation is proceeded to probe the membrane with a secondary antibody for 1 hour. Visualised the protein bands with ECL or ECL select as described above. If the procedure was successful, the membrane was incubated with a new primary antibody at 4℃ overnight again, and the rest of the protocol remained the same as described above.

Image Lab 6.0.1 software was used to quantify relative band intensities by normalizing the integrated density of each sample to the global background.

Product name	Quantity	Brand and Product code
Glycine	15g	G8898, Sigma
SDS	1g	
Tween-20	10ml	P1379, Sigma Aldrich
Elix Water	Fill up to 1L	NA
Adjust PH to 2.2		

Table 2. 10 Quantity of components for Stripping Buffer

2.7 Statistics

All statistical analysis were performed using either Statistical Package for the Social Sciences (SPSS) version 23.0 or GraphPad Prism 9.3.1 Particular statistical tests performed for each, and different experiment are described in the detail chapters. All data presented in either graph or table were shown as Means ± Standard error of the mean (SEM).

Chapter 3 The assessment of iodine intake of UK population using the National Diet and Nutrition Survey Rolling Programme (NDNS RP).

3.1 Introduction

As mentioned in the general introduction, while the global iodine status has generally improved due to iodine fortification programs, the UK has not implemented such measures and lacks a USI program and mandatory fortification. Research done by Phillips (1997) summarised historical evidence, such as the one conducted in 1924, and revealed a high prevalence of iodine deficiency among schoolchildren in the UK, underscoring the necessity for national iodisation efforts. Moreover, the introduction of iodine-enriched cattle feed in the 1930s led to increased iodine levels in milk, especially during winter, contributing to improved iodine intake due to enhanced milk consumption facilitated by the Milk Act (1934) and National Milk Scheme (1940). Despite the initial belief that iodine deficiency is a problem confined to developing countries or regions with iodine-deficient soil, recent studies have highlighted its prevalence in the UK, particularly among children and women of reproductive age. A 2009 study found significant levels of iodine deficiency among 14-to 15-year-old girls in the UK (Vanderpump et al., 2011), prompting the inclusion of UIC measurements in the NDNS since 2013.

In recent years, it had been widely believed that iodine deficiency only affected developing countries and areas with iodine-deficient soil, leading to the promotion of iodination of salt in such regions. However, recent findings have shown that iodine insufficiency is prevalent in the UK and other European countries, especially in more vulnerable groups such as children and women of reproductive age, despite the previously held belief of sufficient iodine levels in these countries (Bath et al., 2013, Bath et al., 2014, Andersson et al., 2007). A cross-sectional survey conducted by Combet et al. (2015) shed light on the lack of awareness regarding iodine, revealing that 64% of the participants had never heard of iodine, with only 12% receiving sufficient information about its importance. Furthermore, Bath et al. (2016) examined UIC in schoolchildren and found median UIC levels of 161 μg/L in winter and 127 μg/L in summer samples, suggesting adequate iodine intake. However, Vanderpump et al. (2011) analyzed UIC in adolescent schoolgirls

and reported a median UIC of 80.1 µg/L (interquartile range: 56.9–109.0), indicating iodine deficiency in the UK population. These findings underscore the pressing need for heightened awareness and targeted interventions to address the burgeoning iodine deficiency issue in the UK.

As no universal salt iodisation program exists in the UK, it relies primarily on natural dietary sources (Table 1.2) and voluntary iodine fortification as mentioned in section 1.2.5. However, the extent of voluntary fortification varies. The variance in iodine content among these iodine contain-ing foods is influenced by several factors, including the iodine content in the soil, farming practices, and season. Hence, it is challenging to provide an accurate estimation of the iodine content in food.

Seafood (fish and seaweed) is the most abundant source of dietary iodine, but milk, eggs, and dairy products are the main sources of iodine for omnivorous diets in industrialised Western countries due to more frequent consumption(Bath et al., 2012, van der Reijden et al., 2017b, Dahl et al., 2004, Pearce et al., 2004, Witard et al., 2022). For instance, a single 200 mL glass of milk in the UK could fulfill up to 57% of the WHO recommended iodine intake for adults, which is set at 150 micrograms (µg) per day. For pregnant women, for whom the WHO recommends an increased intake of 250 µg per day to support fetal development, the same glass of milk could meet approximately 34% of their iodine requirements(Witard et al., 2022). The elevation of milk as a significant iodine source is largely a by-product of common farming practices. These include supplementation of cattle feed with iodine salts and the use of iodine-based disinfectants in dairy farming(Bath and Rayman, 2016, van der Reijden et al., 2017b). Although the direct impact of pasture iodine levels on the iodine content of milk has diminished over time, seasonal variations continue to influence the iodine content in milk, with higher levels typically observed in winter months when cows are more reliant on iodine-supplemented feed than during the summer months when they may graze on less iodine-rich pastures(Flachowsky et al., 2014, Coneyworth et al., 2020).

Farming methods have also come under scrutiny, with concern being raised regarding the iodine content of organic versus conventionally famed milks, both here (Bath et al., 2012, Bath and Rayman, 2020) and the US(Sakai et al., 2022). In both cases organic milk was found to contain much lower concentrations of iodine. Similarly the research conducted by Stevenson et al. (2018) investigated the iodine levels in conventional, organic, and UHT semi-skimmed milk sold at retail stores in the UK. The results showed that organic milk had a 44% lower iodine content than conventional milk, whereas UHT milk had a 27% lower iodine content. However, a study conducted by Soriguer et al. (2011) investigated the iodine levels in cow's milk in Spain. Their findings revealed that the mean iodine concentration in milk increased from 1991 (117±37 µg/L) to 2008 (259±58 µg/L) ($P < 0.001$). Furthermore, it was observed that the iodine concentration was higher in skimmed milk (273±52 µg/L) compared to semi-skimmed milk (254±57 µg/L) or whole milk (251±61 µg/L) due to the fact that iodide is in the aqueous phase.

In recent years, aquaculture has expanded significantly to meet the increasing global demand for seafood fuelled by population growth. This surge has boosted the production of high-value species, such as Atlantic salmon, European seabass, and marine shrimp. The NHS recommends consuming at least two portions of fish per week, including one oily fish, to maintain a healthy diet. Oily fish, which is rich in long-chain omega-3 fatty acids and vitamin D, is promoted for its heart disease prevention benefits(Weichselbaum et al., 2013, Lehmann et al., 2015). However, the focus on omega-3 and vitamin D overshadows fish as equally important contributors to iodine.

Recent studies, such as one done by Sprague et al. (2021) have analysed the iodine content in over 90 wild and farmed fish species available to UK consumers. Their study revealed that wild fish typically have higher iodine levels than their farmed counterparts, and they identified shellfish, marine fish, and freshwater fish in descending order of iodine content (Figure 1.3). Despite this, Sprague et al. (2021) found that seafood contributes only 9.4–18.0% to the UK's reference nutrient intake of iodine, with variations across different demographic groups. Similarly, Dahl et al. (2004) reported that fish and fish

products accounted for only 23-24% of the total iodine intake. However, adherence to health guidelines by consuming two fish servings weekly leads to fluctuating iodine intake due to the varied iodine content of fish.

This variability underscores the importance of studying the fluctuations in iodine intake. Factors such as dietary diversity and different iodine levels in key foods such as milk and fish contribute to inconsistent iodine consumption. Teng et al. (2006a) and Sprague et al. (2021) highlight the variable iodine content in fish, while Sang et al. (2012) and van de Ven et al. (2014) point to the irregular dietary habits among populations that can affect thyroid health. Considering the UK's lack of mandatory iodine fortification, our study aimed to explore the country's iodine status and the extent of intake fluctuations, filling a critical research gap.

Assessment of iodine status using food diary.

Food diaries are a widely used tool for dietary assessment, enabling individuals to record all foods and drinks consumed over a specified period, such as two days (Karvetti and Knuts, 1992), four days (Knudsen et al., 2011), or even a week (Bellisle et al., 1999). These records provide a comprehensive view of eating habits, portion size, and consumption patterns. The NDNS employs four-day food diaries to strike a balance between the need for detailed intake information and the practicality of shorter recording periods, making them a favoured choice. Although methodologies such as the four-day food diary have limitations in capturing habitual dietary behaviours, they offer practical advantages for assessing nutrient intake. Studies indicate that, while food diaries and food frequency questionnaires (FFQs) provide essential data, they may not always accurately represent an individual's habitual nutrient intake because of their snapshot nature and the potential for recall bias or inaccurate portion size estimation (Shim et al., 2014, Bazzano et al., 2002). However, from a practical standpoint, shorter duration food diaries, such as the four-day diary used by the NDNS, are considered more feasible for participants and researchers. They are less burdensome for participants to complete and easier for researchers to analyse, thus facilitating higher response rates (Bailey, 2021).

In addition, although the four-day record provides a brief snapshot of intake, making it less reflective of habitual diet, the structured approach and representative sample size of 1,000 individuals per year from the UK enhances its credibility. Moreover, the selection of participants from PSU were chosen randomly using postcode sectors, further ensuring the representation of diverse dietary practices across the UK.

Despite these limitations, comparative studies validated the usefulness of food diaries. For example, Bingham et al. (1997) confirmed that food diaries could provide nutrient intake estimations comparable to other more extensive dietary assessment methods. Therefore, while acknowledging that the four-day food diary is not the definitive solution for dietary assessment, its utility and representativeness in capturing iodine status among other dietary data in large-scale surveys justifies its continued application within the NDNS framework. Hence, NDNS was selected as the most appropriate source for examining the level of iodine status and investigating fluctuations in iodine intake for this section.

Assessment of iodine status using urine samples.

Urinary iodide is a reliable and affordable indicator of iodine status and is well-accepted worldwide for defining iodine levels within the population. As mentioned in section 1.2.6, several techniques can be used to assess iodine status. Both spot and 24-hour urine samples are commonly used to assess iodine status in the general population. The WHO currently suggests using the median UIC derived from spot urine samples to characterise the iodine status of a population (2013). A spot urine sample requires a single collection and is easily administered by the individual; however, a 24-hour urine sample necessitates the collection of all urine produced over a 24-hour period. Although spot urine samples are convenient to collect, they have limitations, as they do not provide an exhaustive representation of an individual's daily iodine excretion. The iodine concentration in a spot sample may fluctuate based on the recent dietary intake and hydration status. In comparison to spot samples, 24-hour samples are considered more reliable for measuring and

monitoring daily iodine excretion but can be more challenging for participants to complete. A study carried out by Andersen et al. (2008) suggested that in order to assess iodine status at the population level, 500 spot urine samples would be required to estimate status with approximately ±5% precision. For individuals, iodine excretion from spot urine samples reflects the iodine intake of the individual for a short period prior to sample collection (Als et al., 2000). In their conclusion, Andersen claims that for individuals, to prevent misleading assessments, a minimum of ten urine samples from an individual are necessary.

In the NDNS RP, through survey years 1 to 5, the 24-hour urine sample was exclusively used for sodium monitoring. This approach allows for a comprehensive assessment of sodium intake, an important factor in dietary surveys, given its implications for blood pressure and cardiovascular health(Terry et al., 2016). However, starting from year 6, there was a methodological shift from 24-hour to spot urine sample collection. The timing of spot urine sample collection was also standardised; it was performed either during the first interview, which coincided with the distribution of the four-day food diary, or on the day the food diary was collected (third interview). Additionally, spot urine samples were used for urine iodine monitoring (Bates et al., 2014, Page et al., 2016). Spot urine samples have been determined to be a more practical choice for a large research project such as NDNS, as they are easier for participants to provide.

Aim and Hypotheses

The main aim of this study was to provide a comprehensive overview of iodine consumption and iodine status in the UK population across various age groups. Additionally, we aimed to explore the extent of fluctuations in iodine intake, its correlation with iodine status as measured by the UIC, and the contribution of iodine-rich food sources within different fluctuation groups.

We analysed intake levels from NDNS participants during years 9-11 (2016-2018), utilising NDNS-provided spot urine (UIC) data to assess the iodine

status of participants. In addition, we used the daily data provided by the NDNS to summarise the variations in individual dietary intake and assign fluctuation ratings. This was then used to analyse and summarise the correlation between UIC and individual fluctuation ratings across different age groups and genders.

Moreover, we investigated the NDNS food-level data to determine the contribution of various foods to total iodine intake. Using the established fluctuation ratings, we identified variations in food sources contributing to iodine levels among different fluctuation groups.

Thus, this chapter aims to delineate the iodine status and daily intake among the UK population and to uncover differences in food source contributions across fluctuation groups. This could highlight potential interventions to address iodine deficiency or excess due to fluctuating iodine intakes.

We hypothesised that the median UIC of male and female participants would be within an adequate range; potentially, the median UIC for females would be lower than that for males. Additionally, we propose that with fluctuations in daily iodine consumption, we hypothesised that milk and milk-related products contribute the most to iodine consumption; however, when the iodine intake of the participant fluctuates heavily, fish-related food increases its contribution level.

3.2 Method

3.2.1 The National Diet and Nutrition Survey Rolling Programme (NDNS RP) and the survey population.

The NDNS provided all information examined and discussed in this chapter. All data were obtained from the UK Data Archives (NatCen, University of Essex, Colchester, Essex, UK). The UK Ministry of Agriculture, Fisheries and Food and the Department of Health created the National Food and Nutrition Survey in 1990 as a continuous, cross-sectional survey to evaluate the diet, nutrient intake, and nutritional health of the general population in the UK.

The NDNS has been ongoing since 2008 and is a cross-sectional study that uses a sampling frame to select a representative sample of people from the UK population aged 1.5 years and above each year of the survey.

The data selected in the chapter were from the most recent NDNS results, Years 9 to 11(04/2016 to- 6/2019). Public Health England and the UK Food Standards Agency contributed to funding the NDNS during data collection from years 9 to 11 of the NDNS Rolling Program. The sample was taken from Primary Sampling Units (PSUs), which are small geographical areas randomly selected from across the UK using postcode sectors. Subsequently, a list of addresses was randomly selected from each PSU. The survey aimed to collect data from a UK representative core sample of 1,000 people per year, consisting of 500 adults aged 19 years and over and 500 children aged 1.5–18 years. To achieve this, information about the survey's purpose was first posted to all selected addresses, followed by a face-to-face visit to each address by an interviewer to recruit eligible participants.

Up to one adult and one child were invited to participate from each household, and the parent or legal guardian of the child participant gave their written approval. The survey provides quantitative data on food and nutrient intake using dietary data collected through a 4-day food and drink diary, along with lifestyle, physical activity, and characteristics through a face-to-face interview and other related biochemical indices from blood or urine samples. The total number of observations per parameter for each age/sex cohort is not constant

because some NDNS data were missing as a result of lower participation in blood and urine sample collection than for nutritional intake. Details regarding the design of the NDNS can be found in the NDNS user guide (2020). The amount of nutrients consumed from food during the recording of the four-day diary was measured by the UK Nutrient Databank (NDB). The data in the NDB include published UK food composition tables and processed foods. Three main sources provided nutrient data in NDB; Nutrient analysis of foods, food label information from manufacturers, and recipe calculations for home-cooked meals and manufactured products. details of the NDB are listed in Appendix A of the NDNS user guide (2020).

There were a total of 3,558 participants during the NDNS survey from years 9 to 11, in which year 9 included 1253, year 10 had a total of 1211, and year 11 had 1094. All participants completed at least three days of the four-day food diary in the NDNS RP. Eight weeks later, they were interviewed by a trained nurse to collect fasting blood samples, physical measurements, and information on prescribed medicine.

The age cohort and actual participants used in this chapter were based on the provided and available urine samples. Participants who were unable or unwilling to provide urine samples were excluded from this study. The numbers of participants and their age groups are presented in Table 3.1.

Table 3. 1 **The age cohort and actual participants used in the NDNS year9-11 (2020)**

AGE GROUPS	MALE	FEMALE
4-12	391	297
13-18	175	186
19-60	431	635
>60	205	250
19-45		416
TOTAL	1202	1368

3.2.2 Iodine level analysed by the urine iodine concentration (UIC) sample.

Spot urine samples were supplied by participants over the age of four. Samples were collected at the same time as the four-day diary and prior to

blood collection. Details of the spot urine sample collection process can be found in **Appendix M** (2020).

Participants aged ≥ 16 years were requested to provide written consent. For children under the age of 16 years, written consent was obtained from a parent or legal guardian, and, where possible, written assent was obtained from the child. Following the visit, the interviewers were directed to dispatch the collection tube to the Medical Research Council Elsie Widdowson Laboratory (MRC EWL) by post. Samples were stored in a refrigerator at a temperature of 5-8 °C for up to two weeks before being divided into three portions of 1.7 mL of urine each. If the spot urine sample was smaller than 5 mL, the aliquoting process was modified accordingly. The aliquots were stored at -20 °C.

Appendix N provides detailed information regarding the spot urine iodine concentrations. The iodine concentration in spot urine samples was quantified using inductively coupled plasma mass spectrometry (ICP-MS). ICP-MS measured the 127I isotopes present in the samples, which were introduced into the instrument through a flow injection system in conjunction with a sea spray nebuliser and cyclonic spray chamber configuration.

The WHO (2013) standard of UIC level for public health significance was employed to generate sub-groups for the UIC result as shown in table 3.2.

Table 3. 2 **Urinary iodine Concentration (UIC) cut-off values for public health significance indicator and specified rating**

UIC LEVEL(µG/L)	IODINE INTAKE LEVEL	IODINE STATUS	IODINE LEVEL RATING
<20	Insufficient	Severe deficiency	1
20-49	Insufficient	Moderate deficiency	2
50-99	Insufficient	Mild deficiency	3
100-199	Adequate	Adequate iodine nutrition	4
200-299	Above requirements	Slight risk of more than adequate	5
>300	Excessive*	Risk of adverse health consequences	6

Based on WHO recommendations for median UIC in school-age children; applies to adults but not to pregnant and lactation women.
*The term "excessive" here means "in excess of the amount required to prevent and control iodine deficiency."

3.2.3 Assessing fluctuation levels using standard deviation (SD)

To examine the fluctuation of iodine intake within the tested population via the 4-day food diary data from the NDNS, we utilised the standard deviation (SD) of the four-day iodine intake as a measure of fluctuation. Specifically, the SD was calculated for each participant's iodine intake levels (in µg), recorded in the food diary (who finished either three- or four-day food diary).

We employed percentiles of SD as benchmarks to classify the level of fluctuation into distinct categories. According to Table 3.3, the 25th percentile of iodine intake fluctuation (SD) was 27.31, the median (50th percentile) was 43.9, and the 75th percentile was 74.84. We established the fluctuation score by referencing the 25th, 50th, and 75th percentiles of the SD of iodine intake. Thresholds were set at SD levels of 25, 50, and 75. In addition, we introduced a category for more extreme fluctuations, set at an SD level of 100.

Based on this framework, we assigned fluctuation factors to represent varying levels of intake variability, as shown in Table 3.4. A fluctuation factor of 1 was assigned if an individual's SD was < 25, reflecting minimal variability. A factor of 2 was assigned if the SD fell between 25 and 50, indicating mild fluctuations. Moderate fluctuation, represented by a factor of three, corresponded to an SD between 50 and 75. A factor of four, indicating high fluctuation, was assigned an SD between 75 and 100. Lastly, if the SD surpasses 100, the fluctuation factor increases to 5, signifying a very high variability in iodine intake.

Table 3. 3 **Distribution of Iodine Intake Variability Measured by Standard Deviation (SD) Across Percentiles**

Percentiles (%)	SD
20	23.74
25	27.31
40	37.41
50	43.90
60	54.02
75	74.85
80	84.28

Table 3. 4 **Fluctuation score for indicating iodine intake irregularity**

STANDARD DEVIATION (SD) OF FOUR-DAY FOOD DIARY IODINE	IODINE INTAKE FLUCTUATION INDICATOR SCORE	IODINE FLUCTUATION STATUS
<25	1	Stable
25-50	2	Relatively stable
50-75	3	Mild fluctuation
75-100	4	Moderate fluctuation
>100	5	High fluctuation

3.2.4 Contribution of different foods to iodine intake

The NDNS food-level data provided details on the type and quantity of food consumed. The contribution of food groups to the average iodine intake was calculated from the food-level data. We used the variables ‘food name’ and 'food group description’ to identify food groups according to the categories laid out within the iodine-rich food groups (including milk and dairy products, fish and fish products, and eggs).

Person-level daily average intake of micronutrients for iodine was obtained from the NDNS food-level data, which provided information on the type and quantity of food consumed. A pivot table was created to gather iodine intake data from food sources at the personal dietary level. The serial number of the participants, 'seriali', was used to identify participants, while 'MainFoodGroupDesc' represented the food group recorded in the food diary, and 'iodineμg' represented the iodine content of the food recorded in the food diary. (supplement)The iodine content collected in the pivot table was then divided by 'DiaryDaysCompleted', either 3 or 4, to obtain the mean iodine content of the target food. This mean iodine content was then compared with the mean iodine level of the participants to obtain the contribution percentage of the target food.

3.2.5 Data Analysis

Statistical analyses were performed using the SPSS Statistical package (version 23.0. Armonk, NY: IBM Corp, Released 2020). Participant characteristics are presented as mean and standard error of the mean (SEM);

means ± SEM. Participants were excluded from the analysis when a value for UIC was not provided.

The normality of the data was established using Shapiro–Wilks, and an appropriate parametric or non-parametric test was performed. UIC Results of all age groups and food groups indicated non-normally distributed data ($P < 0.05$). Pearson’s correlation was employed to assess the relationship between UIC and fluctuations in dietary iodine intake when variables were normally distributed. Spearman's correlation was employed if not normally distributed. Mann-Whitney & Kruskal-Wallis tests were conducted to assess the differences in daily iodine intake across the various fluctuation rating categories. Participants were excluded from the analysis when a value for UIC was not provided.

3.3 Results

3.3.1 The observation of current UK iodine status using UIC from NDNS

To investigate the fluctuation rate of the UK population, we first conducted an analysis of the iodine status of the population by utilizing the UIC obtained from spot urine samples provided by NDNS. It is noted that among the individuals who participated in NDNS year 9 to 11, a total of 988 (27.77%) did not provide a urine sample for analysis. The NDNS program only collected spot urine samples from children aged four and above, resulting in 306 child participants who were exempt from providing samples. Furthermore, 682 participants (19.17%) did not provide spot urine samples.

The UIC was assessed across different age groups and genders to evaluate iodine status, as presented in table 3.5. In children aged 4-12 years, the median UIC was recorded at 158 μg/L (interquartile range [IQR]: 100–257 μg/L) for males and 132 μg/L (IQR: 79–236 μg/L) for females. Adolescents aged 13-18 years showed median UIC values of 143 μg/L (IQR: 81–213 μg/L) for males and 105 μg/L (IQR: 67.75–170 μg/L) for females. Among adults aged 19-60 years, median UIC levels were 114 μg/L (IQR: 67–178 μg/L) for males and 105 μg/L (IQR: 61–162 μg/L) for females. In the elderly population (above 60 years), the median UIC was 142 μg/L (IQR: 82–199.5 μg/L) for males and 118 μg/L (IQR: 66.75–189.25 μg/L) for females.

Additionally, we conducted tests specifically on female participants in the reproductive age group (19-45 years). The results indicated that the median UIC(IQR) in this age group was 100μg/L (IQR: 54.5.75–157.5 μg/L). This level is considered borderline for adequate iodine status. Furthermore, the analysis showed that 49.9% of the participants in this specific age group were at potential risk of iodine deficiency, highlighting a significant concern for this demographic in terms of iodine nutrition.

Table 3. 5 **UIC status in UK population from NDNS Year 9-11.**

	Male		Female	
Age Groups	Median UIC (µg/L)	Quartile (µg/L) (25%,75%)	Median UIC (µg/L)	Quartile (µg/L) (25%,75%)
4-12	158	(100,257)	132	(79,236)
13-18	143	(81,213)	105	(67.75,170)
19-60	114	(67,178)	105	(61,162)
60+	142	(82,199.5)	118	(66.75,189.25)
18-45			100	(54.5,157.5)

3.3.2 Overall dietary iodine level fluctuation rating and its association with iodine status

Table 3.6 categorizes participants by their iodine intake fluctuation rating, as defined in Section 3.2.3, and the UIC levels of the fluctuation rating groups, indicating their iodine status. The data were separated by gender, providing a clearer understanding of how fluctuation ratings and iodine status differ between males and females.

For males, median UIC ranges from 131.5 µg/L (Stable) to 155 µg/L (High fluctuation). For females, the range was from 103 µg/L (stable) to 121.5 µg/L (high fluctuation).

The Kruskal-Wallis H test showed that there was a statistically significant difference in UIC between the fluctuation groups of females ($p = 0.023$); however, no difference was observed in male ($p = 0.148$).

The percentage of participants in each category varied, with the majority in the' relatively stable' group for both genders.

Additionally, the table reflects the comparison of UIC levels between different fluctuation rating groups, utilizing the Mann-Whitney U test. In males, the median UIC level in the high fluctuation group is significantly higher than that in the stable intake group ($p < 0.05$). Similarly, in females, the median UIC levels in the relatively stable, moderate fluctuation, and high fluctuation groups are significantly higher compared to the stable intake group ($p < 0.05$). This data suggests differences in iodine status with the variability of iodine intake among different fluctuation groups.

Table 3. 6 **UIC status in different fluctuation rating groups from NDNS Year 9-11.**

	Male			Female		
Fluctuation rating	Total participants number N (%)	Median UIC (µg/L)	P value[a]	Total participants number N (%)	Median UIC (µg/L)	P value[a]
1-Stable	214 (17.8%)	131.5	0.148	352 (25.7%)	103	0.023*
2- Relatively stable	386 (32.1%)	136		494 (36.1%)	111[#b]	
3- Mild fluctuation	246 (20.5%)	140.5		238 (17.4%)	110	
4- Moderate fluctuation	153 (12.7%)	132		132 (9.6%)	128[#b]	
5- High fluctuation	203 (16.9%)	155[#b]		152 (11.1%)	121.5[#b]	

a. Kruskal-Wallis tests significant level at *P<0.05

b. Mann-Whitney U test significant level at # P<0.05 comparing to fluctuation rating 1(stable).

3.3.3 Regional comparison iodine status dietary fluctuation

To compare the nutritional status between UK countries, participants were selected from a core UK sample (England), Scotland, Wales, and Northern Ireland (NL) in the NDNS RP study. This allowed us to identify differences in iodine status based on the UIC levels. Among the four countries, female participants from Wales had the lowest median UIC of 89µg/L. The median UIC from all other countries and sex groups was within the adequate iodine status range of 100-150µg/L. The median UIC of male participants in Scotland was highest at 150µg/L. Male respondents from Northern Ireland had the lowest percentage at 24% of potentially insufficient iodine levels (UIC <100µg), whereas female participants from Wales had the highest, at 55.2% (Table 3.7).

To further examine the dietary iodine fluctuations in each region, we analysed the percentage of participants who had stable or highly fluctuating dietary iodine intake and who submitted urine samples. The results of this investigation are presented in Table 3.8.

After careful consideration, we have chosen not to conduct additional regional or country-specific analyses in our study. This decision was made due to the insufficient sample sizes for countries other than England, particularly in Scotland and Wales, where the number of participants of both genders is fewer than 100, without further subdividing them into different age groups.

Table 3. 7 **UIC status in different countries from NDNS yr 9-11**

	Male					Female				
Countries	Total participants number N	Median UIC (µg/L)	Quartile (µg/L) (25%,75%)	Range (µg/L) (Min, Max)	<100µg (%)	Total participants number N	Median UIC (µg/L)	Quartile (µg/L) (25%,75%)	Range (µg/L) (Min, Max)	<100µg (%)
England	884	134	(78,200)	5, 903	35.3%	978	112	(68,186)	4, 66709	43.5%
Scotland	79	150	(90,259)	25, 1045	27.8%	84	120	(68.25,202)	3, 5256	33.3%
NL	150	141.5	(100.75,252.75)	15, 903	24%	201	106	(66.5,202)	4, 780	44.8%
Wales	89	142	(77.5,193)	18, 477	39.3%	105	89	(51,145)	8, 895	55.2%

Table 3. 8 **Fluctuation rating proportion in different countries from NDNS yr 9-11.**

	Male					Female				
	Fluctuation rating (% of regional total participants)					Fluctuation rating (% of regional total participants)				
Countries	1	2	3	4	5	1	2	3	4	5
England	17.3%	31.1%	21.6%	12.6%	17.4%	25.3%	37.2%	17.6%	9.2%	10.7%
Scotland	13.9%	35.4%	22.8%	11.4%	16.5%	28.6%	27.4%	21.4%	8.3%	14.3%
NL	21.3%	32%	16%	17.3%	13.3%	28.4%	36.8%	12.9%	11.4%	10.4%
Wales	20.2%	39.3%	14.6%	7.9%%	18%	22.9%	31.4%	21%	11.4%	13.3%

3.3.4 Assessment of the association between intake variation and absolute intake levels.

In our analysis of the NDNS data for participants in different age groups and genders, we assessed the relationship between UIC and the fluctuation in daily iodine intake, represented by the SD of iodine intake. Due to the non-normal distribution of the data, Spearman's rank correlation was employed.

The scatter plot in our study (Figure 3.1 a) showed no significant correlation between UIC levels and iodine intake fluctuation scores among the age group of 4-12 years old male participants with a Spearman's correlation coefficient (r) of 0.043(p=0.401). This indicates a negligible correlation between the UIC and SD of daily iodine intake, suggesting that variations in iodine intake do not significantly affect UIC levels in this age group. However, the relationship between mean iodine intake and UIC of the male aged 4 to 12 shown in Figure 3.2a has a slight positive correlation, with a Spearman's correlation coefficient (r) of 0.232 ($p<0.001$). Defined by the equation, this suggests a moderate increase in mean iodine intake associated with higher UIC, although with a low coefficient of determination ($R^2 = 0.061256$).

For female participants in the same age cohort, as shown in Figure 3.1b, the Spearman's correlation coefficient for this dataset was 0.163 (p=0.005), suggesting a statistically significant but weak positive relationship between iodine intake fluctuation and UIC. The relationship between mean iodine intake and UIC shown in Figure 3.2b, analysed using the Spearman's correlation coefficient for this data, was r = 0.272, with a p-value of less than 0.0001, indicating a statistically significant moderate positive correlation between mean iodine intake and UIC in this female age group. This suggests that as the UIC increases, indicating higher iodine levels in the urine, the mean iodine intake also tends to be higher.

For adolescent participants aged 13 to 18 years, the analysis of the correlation between UIC and the SD of daily iodine intake revealed Spearman's correlation coefficients of 0.143 (p = 0.06) for males and 0.066 (p = 0.371) for females. These values indicate a lack of significant correlation

between UIC levels and the fluctuation factor (SD) of iodine intake within this age group, as shown in Figures 3.1 c and d. Conversely, the relationship between mean iodine intake and UIC, as shown in Figure 3.2 c and d, shows a different trend. The Spearman's correlation analysis suggests a statistically significant, moderate positive correlation for both genders within this age cohort, with correlation coefficients of 0.365 ($p < 0.001$) for males and 0.163 ($p < 0.05$) for females. This indicates that unlike the fluctuations in iodine intake, the average iodine intake is positively correlated with UIC, suggesting that higher regular iodine intake is associated with higher UIC levels among both male and female adolescents.

For the adult cohort aged 19–60 years, the Spearman's correlation analysis yielded coefficients of 0.126 ($p = 0.009$) for males and 0.038 ($p = 0.338$) for females, indicating a lack of significant correlation between UIC and the SD of daily iodine intake in female but slight significant correlation in male, as displayed in Figure 3.1 e and f. This suggests that fluctuations in daily iodine intake do not significantly affect UIC levels with female this age group. In contrast, the examination of the relationship between mean iodine intake and UIC, as depicted in Figure 3.2 e, revealed a weak yet statistically significant positive correlation for males ($r = 0.169$, $p < 0.001$), implying that higher average iodine intake is associated with higher UIC levels to a small extent. Similarly, for female participants, the correlation between mean iodine intake and UIC remained significant ($r = 0.128$, $p = 0.001$), suggesting that average iodine intake correlate significantly with UIC levels among females within this age range, as shown in Figure 3.2 f.

In the cohort of elderly participants aged over 60 years, the Spearman's correlation analysis revealed coefficients of -0.007 ($p = 0.917$) for males and 0.057 ($p = 0.37$) for females, indicating an absence of significant correlation between UIC and the SD of daily iodine intake, as illustrated in Figure 3.1 g and h. These results suggest that the fluctuations in daily iodine intake do not significantly impact UIC levels in this age group for both genders. Furthermore, analysis of the relationship between mean iodine intake and UIC, as presented in Figure 3.2 g and h, yielded Spearman's correlation

coefficients of 0.201 (p = 0.004) for males and 0.086 (p = 0.174) for females. These findings further support the conclusion that there is no significant correlation between the average daily iodine intake and UIC levels among elderly females, but significantly correlation was found in males, indicating that daily iodine intake fluctuations do not significantly affect the overall iodine status in female of this age cohort.

In all age and gender cohort as shown in Figure 3.3, the Spearman’s correlation analysis revealed a strong coefficient between the mean iodine intake (μg) with the fluctuation in daily iodine intake (SD).For males, Spearman's correlation coefficients were significant and positive across all age groups: 0.524 for ages 4–12, 0.598 for ages 13–18, 0.594 for ages 19–60, and 0.489 for ages above 60(P< 0.001), indicating a statistically significant correlation. Similarly, for females, the correlation coefficients were also significant: 0.496 for ages 4–12, 0.538 for ages 13–18, 0.489 for ages 19–60, and 0.474 for ages over 60(P< 0.001). These results suggest a strong and consistent relationship between mean iodine intake and daily fluctuations across different demographic groups.

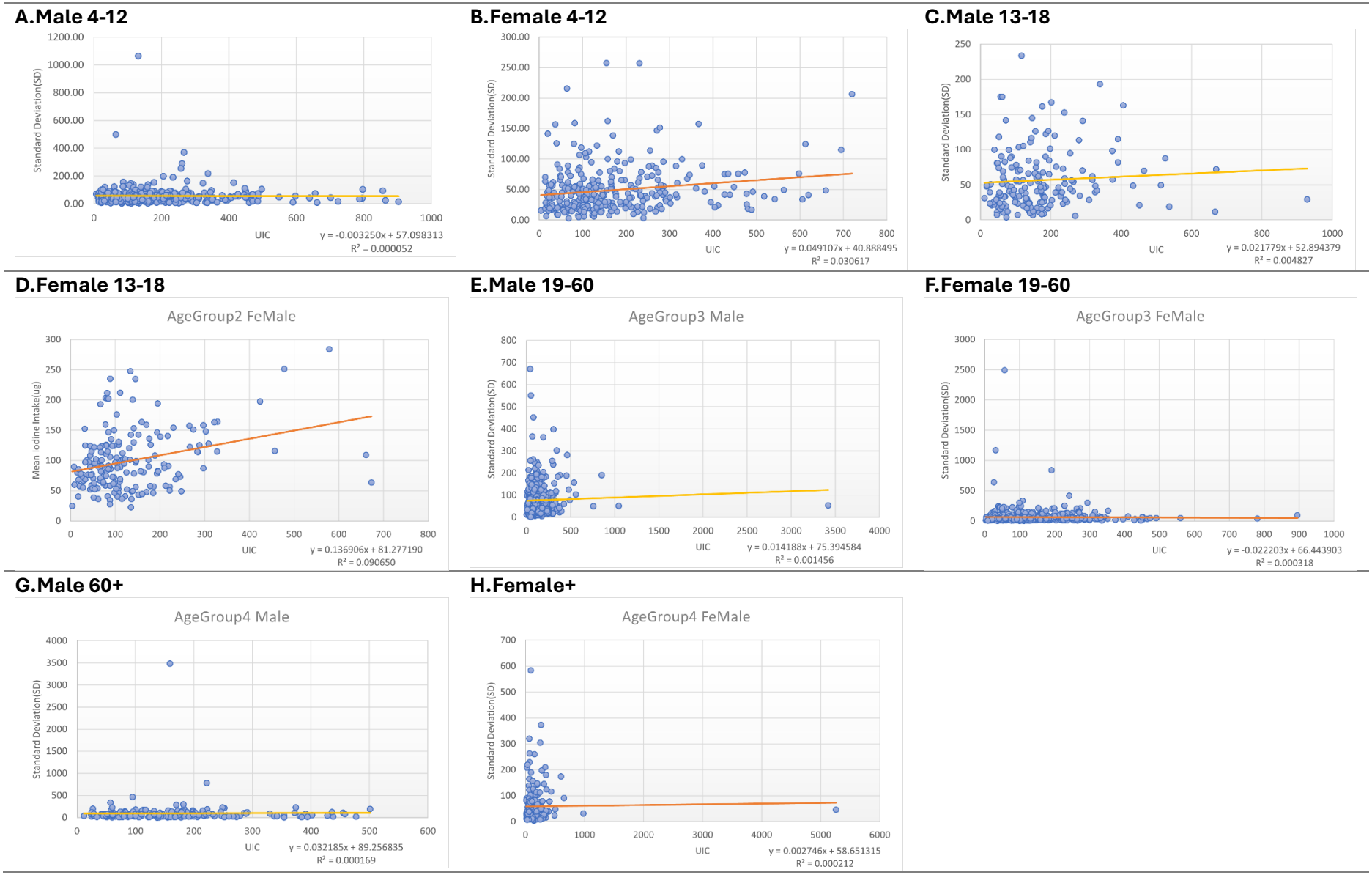


Figure 3. 1 **Correlation Between Urinary Iodine Concentration (UIC) and Standard Deviation (SD) of Daily Iodine Intake in NDNS Participants**

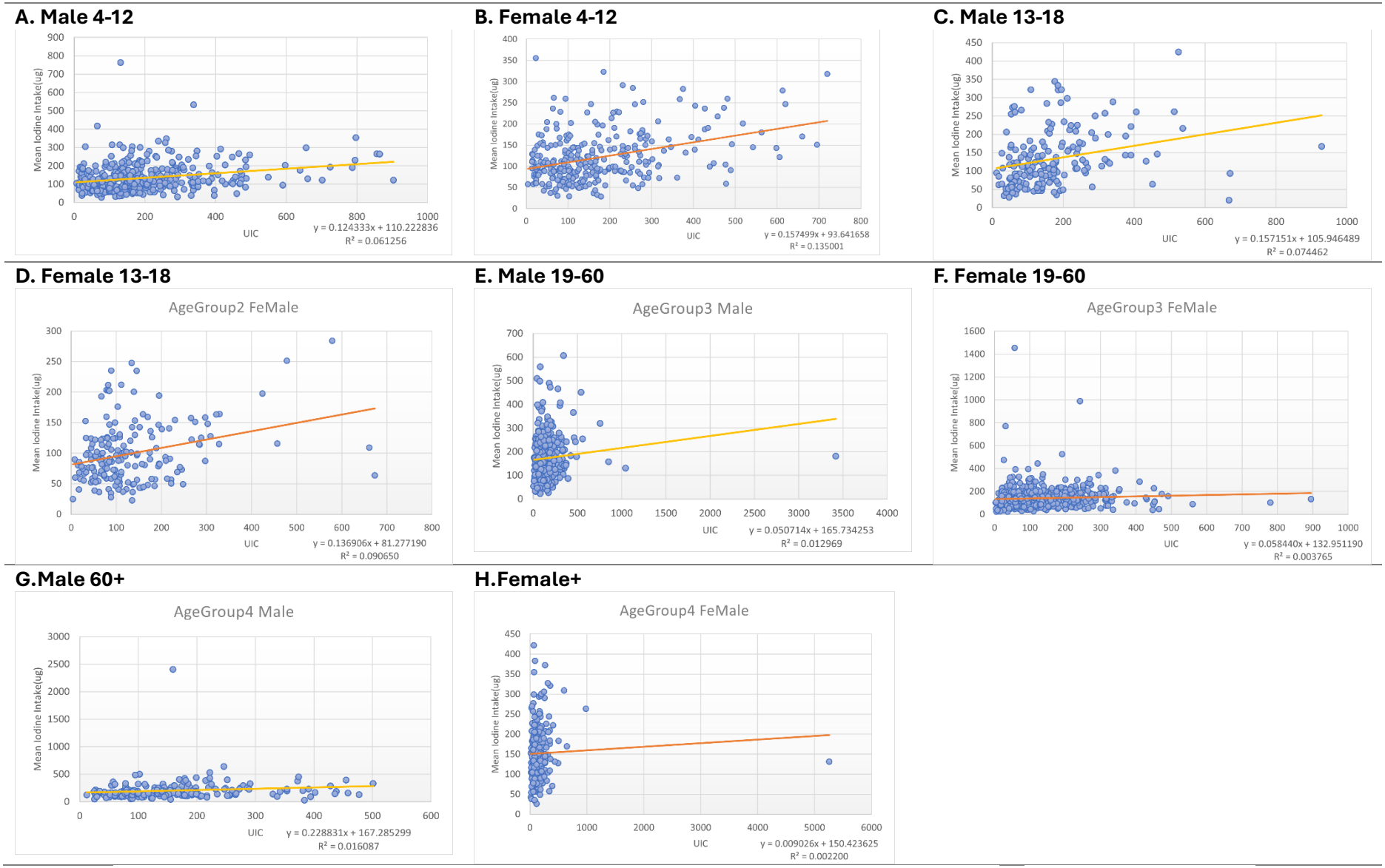


Figure 3. 2 **Correlation Between Urinary Iodine Concentration (UIC) and Mean Daily Iodine Intake(µg) in NDNS Participants**

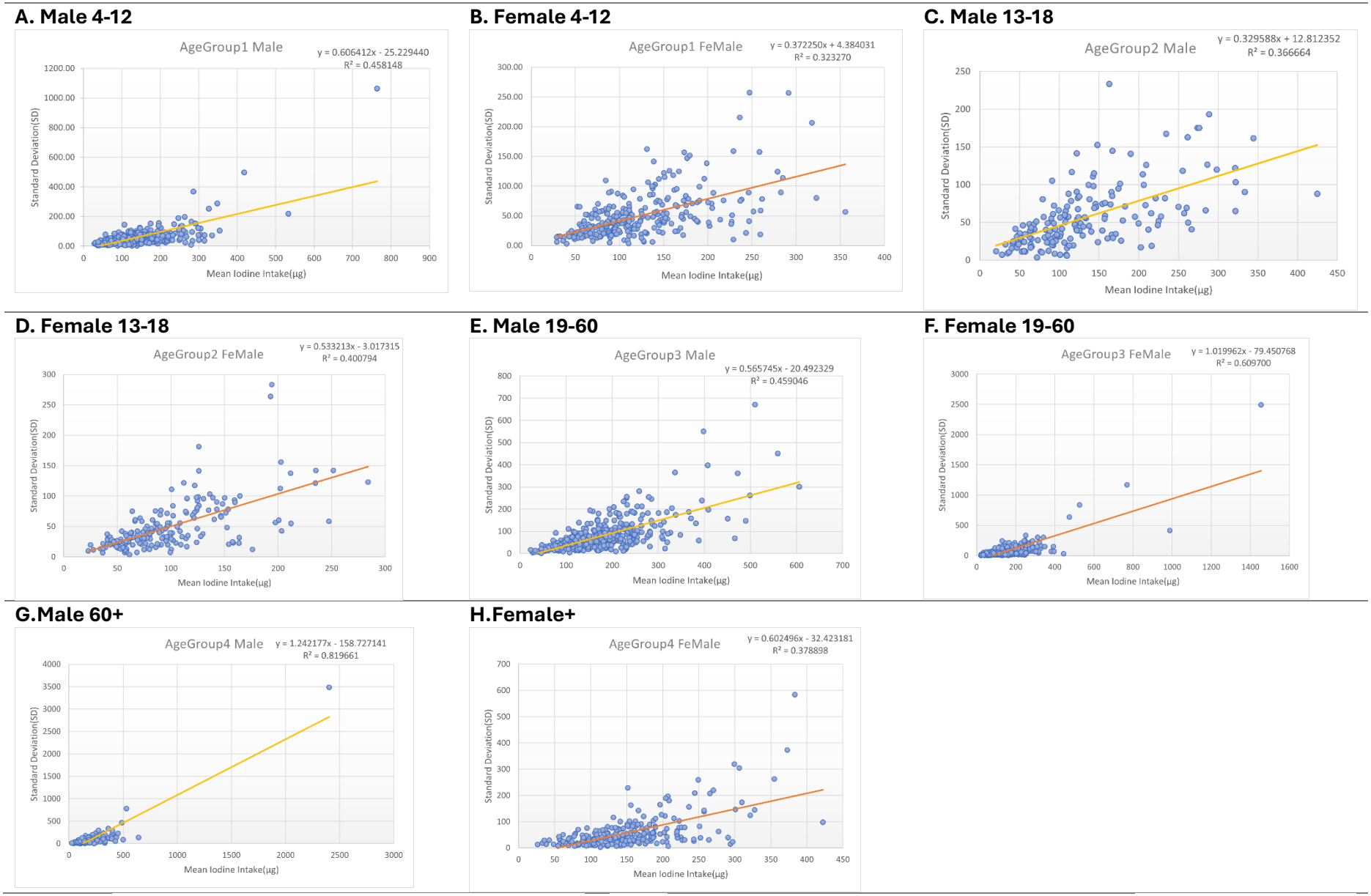


Figure 3. 3 ***Correlation Between Mean Daily Iodine Intake(μg) and Standard Deviation (SD) of Daily Iodine Intake in NDNS Participants***

3.3.5 The composition percentage of different iodine-rich foods in the NDNS four-day food diary in different age groups

The NDNS food categories which had a significant contribution to daily iodine intake were milk, yogurt, cheese, ice cream and others (hereafter referred to as milk and milk products); fish and fish products (including white fish, oily fish, shellfish and others) and egg products.

Age group 4-12 years old.

The majority of iodine consumption among both male and female participants aged 4-12 years was derived from milk and milk products, accounting for 52.19% of males' and 50.52% of females' intake. As shown in Tables 3.9 and 3.10, fish and fish products contributed 9.36% and 9.66% to the overall intake by males and females, respectively. Semi-skimmed milk made the greatest contribution to the proportion of total iodine intake among all milk products, at 21.41% and 21.85% for male and female consumption, respectively. Similarly, Whole milk accounted for 13.3% and 11.53% of male and female milk consumption, respectively.

Although the proportion of daily iodine intake from total milk and milk products did not vary significantly among the fluctuation rating groups for male participants ($p = 0.153$), significant differences were observed in the proportions obtained from cheese ($p = 0.025$), whole milk ($p = 0.004$), and other milk products ($p = 0.017$) across fluctuation groups, as analyzed using the Kruskal-Wallis test (see Table 3.19). Conversely, for female participants, significant differences were noted in the proportion of daily iodine intake from total milk and milk products across fluctuation rating groups ($p = 0.048$), with specific variations noted in one percent milk ($p = 0.028$) and other milk products ($p = 0.036$), as shown in Table 3.10.

Using the Mann-Whitney test to analyze differences between each fluctuation rating group, it was found that in male participants aged 4-12, the proportion of iodine consumed from cheese significantly decreased from 5.07% in fluctuation score 1(stable) to 1.24% in fluctuation score 5(high fluctuation). Conversely, iodine consumption from whole milk significantly increased from

8.6% in fluctuation score 1 to 15.74% in score 3, 17.41% in score 4(moderate fluctuation), and 16.29% in score 5. Additionally, the proportion of iodine from other milk products significantly increased from 2.92% in fluctuation score 1to 12.29% in fluctuation score 5. In female participants of the same age group, the iodine consumption from total milk and milk products significantly increased from 45.51% in fluctuation score 1 to 53.67% and 53.83% in fluctuation scores 2(relatively stable) and 3(mild fluctuation), respectively. However, the percentage contribution reduced back to a level similar to fluctuation score 1 when the fluctuation score reached 5 (46.33%). Additionally, the proportion of iodine from other milk products significantly increased from 1.71% in fluctuation score 1 to 4.84% in fluctuation score 5.

The intake of iodine from fish and fish products showed significant variation among fluctuation rating groups for both male and female participants, with marked statistical significance ($P = 0.0001$) observed for both genders. Notably, in males, significant differences were identified in the intake proportions of oily fish ($p = 0.011$) and white fish ($p < 0.0001$), and in females, a substantial variation was found in white fish intake across different fluctuation rating groups ($p < 0.0001$). The iodine contribution from total fish and fish products markedly increased from 3.53% in males with a fluctuation score of 1 and 3.59% in females with a score of 1 to 15.92% in males with a score of 5 and 25.2% in females with a score of 5. Additionally, the percentage of iodine intake from white fish rose significantly from 1.96% in males and 1.62% in females at fluctuation score 1 to 8.57% in males and 16.64% in females at fluctuation score 5.

For iodine intake from eggs and egg dishes, significant differences were observed across fluctuation rating groups among female participants ($p = 0.032$), indicating variations in consumption patterns. However, for male participants, these differences were not statistically significant ($p = 0.126$), suggesting a more consistent intake among this group.

Table 3. 9 **Percentage contribution of food and food groups to daily iodine intake for male participants aged 4 to12 years. NDNS years 9-11**

Participants years 4-12	All			**Fluctuation rating groups**										K-W
Male	N	**Mean**	**SEM**	**1(n=75)**		**2(n=153)**		**3(n=83)**		**4(n=49)**		**5(n=31)**		**P Value**
Food group				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Milk and milk products	391	52.19	1.05	49.12	2.61	50.45	1.57	54.82	2.19	55.00	2.90	56.68	4.30	0.153
Of which														
Cheese	391	3.16	0.24	5.07	0.88	3.26	0.32	3.03	0.40	1.36	0.21	1.24	0.25	0.025*
Whole Milk	391	13.30	1.08	8.60	2.07	12.36	1.69	15.74$^{\#\#}$	2.37	17.41$^{\#\#}$	3.44	16.29$^{\#}$	4.44	0.004**
Semi Skimmed Milk	391	21.41	1.14	22.61	2.82	20.56	1.80	22.21	2.34	21.15	3.22	20.98	4.34	0.949
Skimmed Milk	391	0.85	0.24	0.58	0.35	1.10	0.45	0.94	0.65	0.21	0.20	1.03	0.76	0.316
One Percent Milk	391	0.59	0.23	0.32	0.32	0.55	0.35	0.00	0.00	1.75	1.30	1.14	0.97	0.097
Yoghurt, fromage frais and other dairy desserts	391	6.31	0.42	6.36	1.13	6.73	0.68	7.10	0.87	6.37	1.30	2.98	0.64	0.216
Ice Cream	391	2.46	0.23	2.59	0.55	2.99	0.42	2.38	0.47	1.81	0.40	0.73	0.28	0.690
Other milk and cream	391	4.02	0.46	2.92	0.72	2.91	0.68	3.41	0.75	4.94	1.36	12.29$^{\#\#}$	2.92	0.017*
Fish and fish products	391	9.36	0.67	3.53	0.83	9.15$^{\#\#\#}$	0.92	9.70$^{\#\#}$	1.48	14.22$^{\#\#\#}$	2.41	15.92$^{\#\#\#}$	3.50	0.0001***
Of which														
Oily Fish	391	0.37	0.08	0.55	0.21	0.33	0.11	0.06	0.03	0.84	0.35	0.19	0.10	0.011*
White fish coated or fried	391	6.70	0.56	1.96	0.73	7.28$^{\#\#\#}$	0.88	7.32$^{\#\#\#}$	1.24	9.96$^{\#\#\#}$	2.11	8.57$^{\#\#\#}$	2.17	0.000***
Other white fish shellfish & fish dishes	391	2.29	0.43	1.03	0.39	1.55	0.39	2.32	0.99	3.43	1.62	7.16$^{\#}$	3.24	0.156
Others	391													
Of which														
Eggs and egg dishes	391	2.21	0.34	4.32	1.59	4.91	1.02	4.36	1.56	5.45	1.53	4.00	1.44	0.126

Kruskal-Wallis tests significant level at *P<0.05; ** P<0.01; ***P<0.001

Mann-Whitney U test significantly different $^{\#}$ p<0.05 $^{\#\#}$P<0.01 $^{\#\#\#}$ P<0.001comparing to fluctuation rating 1.

Table 3. 10 **Percentage contribution of food and food groups to daily iodine intake for female participants aged 4 to12 years. NDNS years 9-11**

Participants years 4-12	All			**Fluctuation rating groups**										K-W
Female	N	Mean	SEM	**1(n=88)**		**2(n=98)**		**3(n=57)**		**4(n=29)**		**5(n=25)**		**P Value**
Food group				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Milk and milk products	297	50.52	1.24	45.51	2.33	53.67$^{\#}$	2.00	53.83$^{\#}$	2.69	52.16	4.47	46.33	4.46	0.048*
Of which														
Cheese	297	2.98	0.21	3.22	0.50	3.13	0.35	3.25	0.41	2.63	0.53	1.50	0.38	0.194
Whole Milk	297	11.53	1.12	9.12	1.98	12.24	2.11	14.91	2.69	10.81	2.93	10.40	3.23	0.254
Semi Skimmed Milk	297	21.85	1.33	18.61	2.38	24.32	2.44	20.95	2.71	25.11	4.68	21.78	4.67	0.432
Skimmed Milk	297	1.26	0.41	1.82	0.93	1.43	0.76	1.21	0.95	0.12	0.12	0.07	0.07	0.969
One Percent Milk	297	0.10	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.18	1.18	0.028*
Yoghurt, fromage frais and other dairy desserts	297	7.08	0.50	7.97	1.00	7.02	0.95	6.93	0.95	7.21	1.39	4.34	1.24	0.603
Ice Cream	297	2.62	0.25	3.06	0.54	2.43	0.40	2.69	0.54	2.08	0.55	2.22	0.68	0.951
Other milk and cream	297	3.05	0.46	1.71	0.55	2.97	0.86	3.89$^{\#}$	1.23	4.21$^{\#}$	1.81	4.84$^{\#\#}$	1.60	0.036*
Fish and fish products	297	9.66	0.84	3.59	0.80	6.03$^{\#\#}$	1.02	12.61$^{\#\#\#}$	1.87	21.12$^{\#\#\#}$	3.43	25.20$^{\#\#\#}$	4.53	0.0001***
Of which														
Oily Fish	297	0.36	0.07	0.48	0.17	0.42	0.13	0.13	0.06	0.38	0.19	0.19	0.11	0.523
White fish coated or fried	297	6.80	0.72	1.62	0.50	3.98$^{\#\#}$	0.86	10.35$^{\#\#\#}$	1.73	16.60$^{\#\#\#}$	3.05	16.64$^{\#\#\#}$	4.22	0.000***
Other white fish shellfish & fish dishes	297	2.50	0.45	1.49	0.57	1.63	0.57	2.12	0.79	4.13	1.94	8.37	3.25	0.590
Others	297													
Of which														
Eggs and egg dishes	297	1.34	0.25	6.18	1.94	6.27	1.59	3.63	1.22	3.78	1.98	1.56	1.56	0.032*

Kruskal-Wallis tests significant level at *P<0.05; ** P<0.01; ***P<0.001

Mann-Whitney U test significantly different $^{\#}$ p<0.05 $^{\#\#}$P<0.01 $^{\#\#\#}$ P<0.001comparing to fluctuation rating 1.

Age group 13-18 years old

As indicated in Tables 3.11 and 3.12, for both male and female participants aged 13-18 years, milk and milk products were the primary sources of iodine intake, contributing 43.46% and 38.31% to total consumption, respectively. Among dairy products, semi-skimmed milk was the largest contributor, accounting for 17.56% of male and 16.93% of female iodine intake. Total fish and fish products contributed 7.44% and 7.74% to the total iodine intake for males and females, respectively. When examining the differences in daily iodine intake from total milk and milk products among fluctuation rating groups through Kruskal-Wallis analysis, a significant variation was observed in males aged 13-18 years ($p = 0.036$); however, in females of the same age group, the difference was not statistically significant ($p = 0.118$). Furthermore, in females, significant differences were noted in the proportions of whole milk ($p = 0.017$) and other milk products ($p = 0.001$) across fluctuation groups. Analysis using the Mann-Whitney test revealed that in this age cohort, the percentage of iodine contribution from total milk and milk products significantly rose from 36.3% at fluctuation score 1 to 55.97% at score 4. In female participants, the iodine contribution from whole milk and other milk products significantly increased from 3.27% and 1.83% at score 1 to 11.3% and 12.58% at score 5, respectively.

The intake of iodine from fish and fish products exhibited notable differences between male and female participants, with significant variances noted ($p = 0.0001$ for males and $p = 0.006$ for females). Specifically, within the male participant group, significant variations were observed across fluctuation rating groups for white fish ($p < 0.001$) and shellfish ($p = 0.023$). Similarly, in the female cohort aged 13-18, a significant difference in white fish consumption was noted ($p = 0.001$). The analysis using the Mann-Whitney test revealed a significant increase in the percentage of iodine contribution from total fish products, increasing from 1.99% in male fluctuation score 1 to 25.23% in score 5. In females, this increase was from 2.41% in fluctuation score 1 to 25.62% in score 5. Furthermore, the proportion of iodine derived from white fish consumption markedly increased from 1.03% in males and

1.17% in females, with a fluctuation score of 1, to 16.08% in males and 10.06% in females with a fluctuation score of 5.

Regarding iodine consumption from eggs and egg-based dishes, a statistically significant fluctuation was detected among male participants across different fluctuation rating groups ($p = 0.006$), as determined using the Kruskal-Wallis test. However, this significant fluctuation was not observed among female participants ($p = 0.265$). Further analysis through the Mann-Whitney test indicated that in males, the percentage of iodine contribution from eggs and egg-based products significantly increased from fluctuation scores 1 to scores 2 and 3, registering at 8.62% and 9.04% respectively (refer to Table 3.11).

Table 3. 11 **Percentage contribution of food and food groups to daily iodine intake for male participants aged 13 to18 years. NDNS years 9-11**

***Participants years** 13-18*	All			**Fluctuation rating groups**										K-W
Male	N	Mean	SEM	**1(n=40)**		**2(n=57)**		**3(n=37)**		**4(n=18)**		**5(n=23)**		**P Value**
Food group				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Milk and milk products	175	43.45	1.72	36.30	3.39	41.71	2.61	45.80	4.12	55.97##	4.22	46.65	5.73	0.036*
Of which														
Cheese	175	3.65	0.33	4.48	0.85	4.57	0.62	2.75	0.64	2.63	0.50	2.16	0.44	0.296
Whole Milk	175	8.63	1.43	3.25	1.59	8.66	2.30	8.93	3.58	11.51	5.25	15.22#	5.16	0.140
Semi Skimmed Milk	175	17.56	1.65	18.88	3.17	13.81	2.73	20.92	4.00	20.39	4.62	16.90	5.19	0.351
Skimmed Milk	175	2.20	0.66	0.05	0.05	3.87#	1.52	1.89	1.44	3.73	2.78	1.13	0.95	0.299
One Percent Milk	175	1.05	0.56	0.00	0.00	1.11	0.90	1.49	1.49	0.00	0.00	2.82	2.82	0.699
Yoghurt, fromage frais and other dairy desserts	175	4.53	0.58	3.77	1.13	4.91	0.99	5.28	1.55	5.93	1.96	2.60	1.00	0.609
Ice Cream	175	1.77	0.33	1.50	0.60	2.59	0.73	1.76	0.68	0.95	0.92	0.90	0.36	0.346
Other milk and cream	175	4.07	0.82	4.38	2.09	2.20	0.95	2.77	1.09	10.84	4.65	4.92	1.59	0.082
Fish and fish products	175	7.44	1.10	1.99	0.77	3.71	1.35	7.01#	1.78	9.54	3.57	25.23###	5.02	0.0001***
Of which														
Oily Fish	175	0.43	0.13	0.18	0.17	0.94	0.35	0.30	0.18	0.03	0.03	0.15	0.11	0.385
White fish coated or fried	175	2.23	0.60	1.03	0.73	2.43	1.19	4.25	1.52	7.18#	2.90	16.08###	4.38	0.000***
Other white fish shellfish & fish dishes	175	4.77	0.90	0.79	0.31	0.34	0.15	2.46	0.95	2.33	1.46	9.01	3.83	0.023*
Others	175													
Of which														
Eggs and egg dishes	175	2.03	0.32	2.73	0.89	8.62#	1.66	9.04##	1.93	5.41#	1.39	2.27	0.87	0.006**

Kruskal-Wallis tests significant level at *P<0.05; ** P<0.01; ***P<0.001

Mann-Whitney U test significantly different # p<0.05 ##P<0.01 ### P<0.001comparing to fluctuation rating 1(stable).

Table 3. 12 **Percentage contribution of food and food groups to daily iodine intake for female participants aged 13 to18 years. NDNS years 9-11**

Participants years 13-18	All			**Fluctuation rating groups**										K-W
Female	N	Mean	SEM	**1(n=56)**		**2(n=64)**		**3(n=28)**		**4(n=22)**		**5(n=16)**		**P Value**
Food group				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Milk and milk products	186	38.31	1.74	37.35	3.96	37.01	2.49	34.62	4.06	47.85#	4.11	40.26	5.63	0.118
Of which														
Cheese	186	3.75	0.35	4.63	0.87	3.75	0.53	3.14	0.73	3.31	0.69	2.36	0.72	0.785
Whole Milk	186	4.68	0.93	3.27	1.59	5.30	1.59	3.86	2.18	2.71	2.65	11.30##	4.09	0.017*
Semi Skimmed Milk	186	16.93	1.45	21.23	3.07	13.86	2.18	17.10	3.65	19.10	4.29	10.91	3.46	0.299
Skimmed Milk	186	0.97	0.33	1.96	0.94	0.61	0.38	0.10	0.07	0.87	0.59	0.61	0.61	0.279
One Percent Milk	186	0.48	0.40	0.00	0.00	1.41	1.17	0.00	0.00	0.00	0.00	0.00	0.00	0.216
Yoghurt, fromage frais and other dairy desserts	186	3.92	0.64	2.37	0.87	6.45	1.50	3.70	1.33	2.08	0.84	2.11	1.37	0.275
Ice Cream	186	2.02	0.45	2.07	0.73	2.85	1.07	1.51	0.73	1.29	0.54	0.40	0.28	0.584
Other milk and cream	186	5.56	0.95	1.83	1.08	2.78	0.87	5.21	2.20	18.47##	4.81	12.58##	4.34	0.001**
Fish and fish products	186	7.74	1.19	2.41	0.70	3.48	0.92	13.76#	3.67	12.99	4.83	25.62##	7.37	0.006**
Of which														
Oily Fish	186	0.45	0.11	0.47	0.20	0.39	0.18	0.41	0.24	0.74	0.42	0.30	0.24	0.556
White fish coated or fried	186	2.71	0.66	1.17	0.60	1.34	0.72	10.14	3.31	11.58##	4.65	10.06#	5.46	0.001**
Other white fish shellfish & fish dishes	186	4.57	0.97	0.77	0.28	1.76	0.64	3.21	1.64	0.68	0.44	15.26#	5.86	0.086
Others	186													
Of which														
Eggs and egg dishes	186	2.90	0.48	5.71	1.37	7.69	1.48	8.41	2.86	3.98	1.39	3.92	1.53	0.265

Kruskal-Wallis tests significant level at *P<0.05; ** P<0.01; ***P<0.001

Mann-Whitney U test significantly different # p<0.05 ##P<0.01 ### P<0.001comparing to fluctuation rating 1(stable).

Age group 19-60 years old

In the adult cohort aged 19 to 60 years, milk and milk products emerged as the primary sources of iodine intake for both genders, contributing to 34.28% of male and 38.6% of female dietary iodine respectively. Among dairy products, semi-skimmed milk had the largest impact on total iodine consumption, accounting for 16.09% of male and 15.21% of female intake. Fish and fish products contributed 7.44% to male and 8.48% to female iodine intake. Additionally, males and females consumed an average of 8.19% and 9.21% of their iodine from eggs and egg-based dishes, respectively (Table 3.13 and 14).

Fluctuations in iodine consumption, as analyzed using the Kruskal-Wallis test, revealed significant differences in daily iodine intake from total milk and milk products among the fluctuation groups for both genders ($p = 0.011$ for males and $p < 0.001$ for females). Moreover, the iodine intake from cheese showed significant variability between fluctuation groups for both males ($p = 0.035$) and females ($p < 0.001$). In females, the contribution of skimmed milk to iodine intake also varied significantly between fluctuation groups ($p = 0.002$). Using the Mann-Whitney test to assess differences between specific fluctuation groups, it was observed that in males of this age range, the percentage contribution of iodine from total milk and milk products significantly declined from 35.64% in fluctuation score 1 to 28.19% in score 5. Meanwhile, iodine contribution from cheese increased significantly in scores 2 and 3 (4.65% and 4.09%, respectively) compared to other groups. In contrast, female participants saw a significant reduction in the percentage of iodine contribution from total milk and milk products, from 39.47% in fluctuation score 1 to 30.39% in score 5. Additionally, the contribution from cheese significantly decreased from 4.79% in fluctuation score 1 to 1.77% in score 5, while the contribution from skimmed milk was notably higher in scores 3 and 5 (5.93% and 3.56%, respectively) compared to other fluctuation groups

Table 3. 13 **Percentage contribution of food and food groups to daily iodine intake for male participants aged 19 to 60 years. NDNS years 9-11**

Participants years 19-60	All			**Fluctuation rating groups**										K-W
Male	N	Mean	SEM	**1(n=65)**		**2(n=117)**		**3(n=87)**		**4(n=63)**		**5(n=99)**		**P Value**
Food group				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Milk and milk products	431	34.28	1.02	35.64	3.02	35.84	1.86	35.05	2.18	38.46	2.72	28.19	1.97	0.011*
Of which														
Cheese	431	3.30	0.26	2.58	0.51	4.65#	0.63	4.09#	0.74	2.22	0.35	2.15	0.28	0.035*
Whole Milk	431	5.55	0.70	5.97	1.83	5.40	1.28	3.77	0.98	7.64	2.31	5.70	1.61	0.923
Semi Skimmed Milk	431	16.09	0.90	17.21	2.65	15.93	1.62	17.93	2.12	19.85	2.70	11.52	1.37	0.256
Skimmed Milk	431	2.26	0.38	1.82	0.89	2.45	0.87	2.02	0.79	1.93	0.77	2.75	0.77	0.492
One Percent Milk	431	0.38	0.18	0.00	0.00	0.31	0.31	0.11	0.11	0.95	0.95	0.57	0.34	0.536
Yoghurt, fromage frais and other dairy desserts	431	3.98	0.38	4.92	1.32	4.55	0.79	4.71	0.88	2.88	0.74	2.74	0.56	0.77
Ice Cream	431	0.48	0.11	0.29	0.16	0.87	0.34	0.49	0.25	0.20	0.11	0.30	0.12	0.613
Other milk and cream	431	2.24	0.38	2.85	1.25	1.67	0.48	1.92#	0.62	2.78	1.16	2.47	0.99	0.186
Fish and fish products	431	7.44	1.10	3.39	0.76	3.35	0.61	5.25	1.22	8.71#	1.81	23.07###	2.22	0.000***
Of which														
Oily Fish	431	0.43	0.13	1.46	0.54	0.91	0.23	1.62	0.53	2.22	1.06	1.21	0.38	0.902
White fish coated or fried	431	2.23	0.60	0.27	0.19	1.37	0.51	1.67	0.68	3.21#	1.08	12.06###	1.78	0.000***
Other white fish shellfish & fish dishes	431	4.77	0.90	1.67	0.54	1.06	0.25	1.97	0.78	3.28#	1.00	9.81##	1.85	0.001***
Others														
Of which														
Eggs and egg dishes	431	8.19	0.32	6.58	1.61	8.42#	1.07	10.86##	1.27	6.95	0.93	7.40##	0.85	0.009**

Kruskal-Wallis tests between all fluctuation groups, significant level at *P<0.05; ** P<0.01; ***P<0.001

Mann-Whitney U test significantly different # p<0.05 ##P<0.01 ### P<0.001comparing to fluctuation rating 1(stable).

Table 3. 14 **Percentage contribution of food and food groups to daily iodine intake for female participants aged 19 to60 years. NDNS years 9-11**

Participants years 19-60	All			**Fluctuation rating groups**										K-W
Female	N	Mean	SEM	**1(n=142)**		**2(n=249)**		**3(n=107)**		**4(n=60)**		**5(n=77)**		**P Value**
Food group				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Milk and milk products	635	38.60	0.80	39.47	1.72	40.84	1.19	40.05	2.05	35.15	2.35	30.39$^{\#\#}$	2.42	0.000***
Of which														
Cheese	635	3.82	0.20	4.79	0.53	4.25	0.33	3.70	0.48	2.59	0.39	1.77$^{\#\#\#}$	0.26	0.000***
Whole Milk	635	5.53	0.55	5.59	1.22	6.30	0.91	6.51	1.62	4.88	1.40	2.04	0.77	0.384
Semi Skimmed Milk	635	15.21	0.73	15.11	1.51	17.04	1.23	13.14	1.68	13.43	2.12	13.77	1.96	0.57
Skimmed Milk	635	3.12	0.40	1.98	0.71	2.78	0.63	5.93$^{\#\#}$	1.31	1.68	0.81	3.56$^{\#\#}$	0.99	0.002**
One Percent Milk	635	0.57	0.20	1.03	0.59	0.52	0.26	0.14	0.14	0.00	0.00	0.95	0.92	0.683
Yoghurt, fromage frais and other dairy desserts	635	7.09	0.43	7.93	1.15	7.01	0.64	7.06	0.96	7.82	1.54	5.29	0.81	0.98
Ice Cream	635	0.66	0.10	0.52	0.16	0.69	0.16	0.74	0.29	1.18	0.50	0.33	0.12	0.839
Other milk and cream	635	2.59	0.28	2.53	0.66	2.25	0.37	2.84	0.66	3.57	1.18	2.67	0.83	0.977
Fish and fish products	635	8.48	0.57	3.40	0.46	3.87	0.47	7.15$^{\#}$	0.99	12.30$^{\#\#\#}$	1.99	31.65$^{\#\#\#}$	2.54	0.000***
Of which														
Oily Fish	635	1.27	0.13	1.60	0.30	0.94	0.17	1.11	0.27	2.30	0.73	1.13	0.32	0.398
White fish coated or fried	635	3.74	0.43	0.33	0.19	1.32$^{\#}$	0.34	3.62$^{\#\#\#}$	0.87	5.51$^{\#\#\#}$	1.56	16.63$^{\#\#\#}$	2.40	0.000***
Other white fish shellfish & fish dishes	635	3.48	0.39	1.48	0.29	1.60	0.29	2.42	0.59	4.49	1.34	13.89$^{\#\#\#}$	2.41	0.000***
Others	635													
Of which														
Eggs and egg dishes	635	9.21	0.45	8.27	1.13	10.63$^{\#\#}$	0.75	9.89$^{\#\#}$	1.01	9.02$^{\#}$	1.32	5.56	0.83	0.001***

Kruskal-Wallis tests significant level at *P<0.05; ** P<0.01; ***P<0.001

Mann-Whitney U test significantly different $^{\#}$ p<0.05 $^{\#\#}$P<0.01 $^{\#\#\#}$ P<0.001comparing to fluctuation rating 1(stable).

The contribution of iodine intake from fish and fish products was significantly In the 19-60 age cohort, significant differences were observed in the contribution of iodine intake from fish and fish products between fluctuation groups for both male and female participants ($p < 0.001$). For both genders, contributions from white fish and shellfish to iodine intake varied significantly across fluctuation rating groups ($p < 0.001$). Specifically, the contribution from total fish and fish products significantly increased from 3.39% in males and 3.4% in females at fluctuation score 1, to 23.07% in males and 31.65% in females at score 5. The iodine contribution from white fish also showed a significant increase from 0.27% for males and 0.33% for females at fluctuation score 1 to 12.06% for males and 16.63% for females at score 5. Additionally, the contribution from shellfish significantly rose from 1.67% for males and 1.48% for females at fluctuation score 1 to 9.81% for males and 13.89% for females at score 5.

Furthermore, fluctuations in iodine intake from eggs and egg-based dishes among fluctuation groups were statistically significant for both genders ($p = 0.009$ for males, $p = 0.001$ for females). The percentage contribution from eggs and egg-based meals significantly increased at fluctuation scores 2 and 3 for males (8.42% and 10.86%, respectively) compared to all other groups. In contrast, female participants within this age cohort exhibited a significant decrease in the contribution from eggs and egg-based dishes at fluctuation score 5 (5.56%) compared to other fluctuation scores.

Age group over 60 years old

In individuals over 60 years, milk and milk products were the primary sources of iodine intake for both genders, comprising 38.14% of male and 42.18% of female dietary iodine, respectively. Within dairy products, semi-skimmed milk contributed most significantly to iodine intake, accounting for 18.59% and 19.81% of the consumption in males and females, respectively. Additionally, the average iodine intake from eggs and egg-based dishes was 7.90% for males and 8.34% for females, as detailed in Tables 3.15 and 3.16.

Fluctuations in iodine consumption, assessed using the Kruskal-Wallis test, showed significant differences in daily iodine intake from total milk and milk products among female participants across fluctuation groups ($p < 0.001$). However, for males within the same age group, these differences were not statistically significant ($p = 0.212$).

Further analysis with the Mann-Whitney test on specific fluctuation groups revealed that in male participants of this age cohort, the percentage contribution of iodine from total milk and milk products significantly reduced to 33.36% in fluctuation score 4 compared to other groups. Additionally, the contribution from semi-skimmed milk markedly decreased to 13.48% in fluctuation score 5 from 25.12% in score 1. In female participants, a significant reduction was observed in the contribution of iodine from total milk and milk products, dropping to 29.88% in fluctuation score 5 from an initial 49.85% in score 1; similarly, the contribution from semi-skimmed milk significantly decreased to 13.64% in fluctuation score 5 from 24.75% in score 1.

In this age cohort, significant disparities were observed in the consumption of iodine from fish and fish products among fluctuation groups for both genders ($p < 0.001$). Furthermore, significant variations were noted in the iodine contributions from white fish and shellfish between the fluctuation rating groups for both males and females ($p < 0.001$). Specifically, the share of iodine intake from total fish products significantly increased from 2.92% for males and 3.27% for females in fluctuation group 1 to 25.36% for males and 40.45% for females in group 5. Moreover, the proportion of iodine intake from white fish also showed a significant rise from 0.84% in males and 0.33% in females with a fluctuation rating of 1, to 12.61% in males and 15.33% in females with a rating of 5. Likewise, the iodine contribution from shellfish exhibited a similar increasing trend, from 0.78% in males and 0.98% in females in fluctuation group 1 to 11.17% in males and 24.04% in females in fluctuation group 5.

Table 3. 15 **Percentage contribution of food and food groups to daily iodine intake for male participants aged over 60 years. NDNS years 9-11**

Participants years over 60	All			**Fluctuation rating groups**										K-W
Male	N	Mean	SEM	**1(n=34)**		**2(n=59)**		**3(n=39)**		**4(n=23)**		**5(n=50)**		**P Value**
Food group				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Milk and milk products	205	38.14	1.23	43.13	2.66	37.38	2.04	40.47	2.88	33.36$^{\#}$	3.96	36.02	2.77	0.212
Of which														
Cheese	205	2.71	0.24	2.70	0.48	3.54	0.55	2.61	0.59	3.37	0.94	1.51	0.22	0.202
Whole Milk	205	4.87	0.93	7.42	2.95	1.23	0.50	5.01	2.28	7.53	4.08	6.09	1.84	0.89
Semi Skimmed Milk	205	18.59	1.30	25.12	3.51	19.75	2.02	19.82	3.38	14.99$^{\#}$	3.16	13.48$^{\#}$	2.68	0.053
Skimmed Milk	205	4.65	0.85	1.95	1.65	4.21	1.52	6.09	2.53	2.59	1.62	6.83$^{\#\#}$	1.77	0.066
One Percent Milk	205	0.23	0.19	0.00	0.00	0.16	0.16	0.00	0.00	0.00	0.00	0.76	0.76	0.773
Yoghurt, fromage frais and other dairy desserts	205	4.54	0.64	2.76	0.88	6.59	1.59	4.29	0.99	2.98	1.25	4.24	1.41	0.465
Ice Cream	205	0.96	0.17	1.42	0.51	0.94	0.39	1.16	0.37	0.76	0.28	0.59	0.18	0.681
Other milk and cream	205	1.59	0.40	1.76	0.59	0.95	0.19	1.48	0.52	1.13	0.47	2.52	1.51	0.472
Fish and fish products	205	11.98	1.11	2.92	1.05	8.46$^{\#\#}$	1.47	6.56$^{\#}$	1.42	14.51$^{\#\#}$	3.43	25.36$^{\#\#\#}$	2.89	0.000***
Of which														
Oily Fish	205	2.58	0.46	1.30	0.63	4.30	1.30	2.72	0.95	1.94	0.76	1.58	0.59	0.3
White fish coated or fried	205	4.73	0.82	0.84	0.84	1.63	0.65	2.65	1.07	4.86	2.84	12.61$^{\#\#\#}$	2.53	0.000***
Other white fish shellfish & fish dishes	205	4.67	0.77	0.78	0.37	2.52$^{\#}$	0.68	1.19	0.60	7.71$^{\#\#}$	2.39	11.17$^{\#\#}$	2.56	0.000***
Others	205													
Of which														
Eggs and egg dishes	205	7.90	0.64	6.62	1.45	10.61	1.46	7.42	1.33	11.54$^{\#}$	1.73	4.28	0.83	0.007**

Kruskal-Wallis tests significant level at *P<0.05; ** P<0.01; ***P<0.001

Mann-Whitney U test significantly different $^{\#}$ p<0.05 $^{\#\#}$P<0.01 $^{\#\#\#}$ P<0.001comparing to fluctuation rating 1.

Table 3. 16 Percentage contribution of food and food groups to daily iodine intake for female participants aged over 60 years. NDNS years 9-11

Participants years over 60	All			**Fluctuation rating groups**										K-W
Female	N	Mean	SEM	**1(n=66)**		**2(n=83)**		**3(n=46)**		**4(n=21)**		**5(n=34)**		**P Value**
Food group				Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Milk and milk products	250	42.18	1.21	49.85	1.99	43.14#	2.03	38.56##	3.00	42.13	4.53	29.88###	2.93	0.000***
Of which														
Cheese	250	3.03	0.21	3.21	0.50	3.51	0.38	2.64	0.31	1.75	0.44	2.78	0.51	0.305
Whole Milk	250	3.83	0.70	5.30	1.78	3.09	1.09	4.27	1.19	2.88	1.96	2.76	1.84	0.459
Semi Skimmed Milk	250	19.81	1.26	24.75	2.68	20.71	2.18	16.72	2.73	17.43	4.79	13.64#	2.49	0.181
Skimmed Milk	250	4.10	0.71	3.48	1.45	5.14	1.29	5.21	1.94	4.01	2.34	1.31	0.71	0.698
One Percent Milk	250	0.46	0.26	0.30	0.30	1.01	0.72	0.27	0.27	0.00	0.00	0.00	0.00	0.678
Yoghurt, fromage frais and other dairy desserts	250	7.34	0.60	8.19	1.40	7.46	0.97	6.29	1.50	9.38	1.64	5.58	1.06	0.469
Ice Cream	250	1.18	0.21	0.98	0.27	0.77	0.20	0.72	0.33	3.35#	1.57	1.83	0.85	0.152
Other milk and cream	250	2.44	0.40	3.64	1.07	1.45	0.39	2.45	1.01	3.3	1.26	1.98	0.92	0.444
Fish and fish products	250	11.89	1.05	3.27	0.55	6.67	0.96	12.70###	2.02	11.65#	3.25	40.45###	3.51	0.000***
Of which														
Oily Fish	250	2.10	0.27	1.96	0.40	2.24	0.54	2.92	0.74	1.89	0.74	1.08	0.50	0.215
White fish coated or fried	250	4.03	0.67	0.33	0.23	1.66	0.59	4.75#	1.72	5.10###	1.96	15.33###	3.29	0.000***
Other white fish shellfish & fish dishes	250	5.76	0.88	0.98	0.29	2.77#	0.64	5.03##	1.35	4.65	2.95	24.04###	4.58	0.000***
Others	250										A			
Of which														
Eggs and egg dishes	250	8.34	0.59	6.91	1.25	9.29#	1.05	9.85#	1.39	11.36#	2.10	4.88	0.92	0.007**

Kruskal-Wallis tests significant level at *P<0.05; ** P<0.01; ***P<0.001

Mann-Whitney U test significantly different # p<0.05 ##P<0.01 ### P<0.001comparing to fluctuation rating 1.

Regarding iodine intake from eggs and egg-based dishes, a statistically significant difference was observed among all fluctuation groups for both males and females ($p = 0.007$). The proportion of iodine intake from eggs and egg-based products in males was notably higher at fluctuation score 4 (11.54%) compared to all other fluctuation groups. A similar pattern was observed in females, where fluctuation score 4 exhibited a significant increase in the proportion of iodine intake from eggs and egg dishes (11.36%).

3.3.6 Seaweed consumption among NDNS respondents.

Seaweed has long been a staple in Japanese cuisine and is widely enjoyed across East Asian nations such as Korea and China. Its use in a variety of dishes, including sushi, miso soup, and salads, and as a flavourful base for soups, has contributed to its distinguished presence in regional gastronomies. Recently, the popularity of seaweed has expanded to Western countries, driven by the rising interest in plant-based, vegetarian, and vegan diets. According to the NDNS database records, seaweed intake during years 9-11 was typically classified under 'vegetable not raw' or 'miscellaneous' categories. However, a specific section to record seaweed consumption frequency was introduced in the NDNS RP questionnaire.

Data from the NDNS years 9-11 dataset, as summarised in Table 3.17, show that 13.91% (n=495) of participants reported regular seaweed consumption. To further investigate the frequency and age distribution of individuals who reported consuming seaweed, we divided participants into distinct age groups. We categorised the age groups into children and teenagers (under 18 years old), adults (18-60 years old), and seniors (over 60 years old). As shown in Table 3.18, there were four different frequency levels: consuming seaweed once a month, 1-3 times a month, 1-2 times a week, and more than 3 times a week.

The frequency of seaweed intake varies across different age and gender groups, revealing unique dietary trends. In the under-18 age group, both

males and females reported a moderate frequency, typically consuming seaweed 1-3 times per month. Adults aged 19 to 60 years exhibited a notable increase in seaweed consumption, with females leading the charge; notably, 120 females reported consuming seaweed 1-3 times per month, surpassing 82 males in the same category. This demographic also has a significant portion of indulging in seaweed 1-2 times per week, and some even more than three times per week, showcasing a greater dietary inclusion of seaweed compared to younger individuals. For those over 60, despite smaller sample sizes, there was a persistent trend of sustained or increased frequency of seaweed consumption, with a few individuals consuming it more than three times per week. In summary, the analysis indicated an upward trend in seaweed consumption with age, with females generally exhibiting higher consumption frequencies than males.

Age	**Sex**	**Total**	**Once per month**	**1-3 per month**	**1-2 per week**	**3+ per week**
<18	Male	25	49	23	3	
	Female	29	51	20	6	
19-60	Male	53	82	26	5	1
	Female	49	120	38	10	2
60+	Male	10	18	4	1	1
	Female	15	25	7	2	1
Total	Male	213	149	53	9	2
	Female	282	196	65	18	3

Table 3. 17 **Frequency distribution of seaweed consumer separated by gender, age groups and survey years.**

3.4 Discussion

The objective of this research was to provide an overall picture of iodine status in the UK population across different age groups and regions, and to examine the magnitude of variation in intake levels among participants. Specifically, we analysed the data provided in the NDNS survey for participants in their newest report for years 9-11 (2016-2018) program.

Iodine status from NDNS

First, our examination of UIC data from NDNS revealed that based on spot urine samples, the UK population generally exhibits a relatively adequate iodine status. According to the median UIC values, most males and females across various age groups fell within the adequate range (Table 3.5). However, it is notable that the median UIC levels for female participants are consistently lower than those for males, particularly highlighting that female adolescents and adults are at a higher risk of iodine insufficiency. Specifically, the median UIC for females in the reproductive age group (19-45 years) the median UIC is at the borderline of an adequate level, 100μg/L. Additionally, nearly half (49.9%) of this demographic fell below the recommended UIC threshold.

When Studying the UIC data from a regional point of view, as shown in section 3.3.3, the median UIC from spot urine samples was classified as adequate for participants from England, Scotland, and Northern Ireland, except for females from Wales, who were classified as mildly deficient (median UIC at 89μg/L). Despite regional variations, our study did not delve into detailed country-specific analyses primarily due to limited sample sizes from regions outside England, particularly in Scotland and Wales, where participant numbers for both genders were below 100.

From a gender-specific point of view, NDNS clarified that their research did not include pregnant or lactating females. Which is one of the drawbacks of this study, due to the lack of the presence of such population in NDNS database. In addition, if we set the age group to the reproductive female age

group, the median UIC was borderline adequate for that specific population group. The absence of data from these groups presents a notable disadvantage, as they are especially susceptible to iodine deficiency due to their increased iodine requirements during pregnancy and lactation for fetal and neonatal development (Zimmermann, 2009). A study conducted by Bath et al. (2014) also proved that UK pregnant women are not meet the WHO iodine requirements of the pregnancy median UIC at 85.3ug/L. Many studies have suggested UK females pregnant women not has been facing the problem of iodine deficiency (Vanderpump et al., 2011, Jiang et al., 2019) and that future work or health intervention is needed to help gain more understanding as well as to identify potential solutions to the problem. The lack of information from these populations restricts the scope of the NDNS findings on iodine status and prevents a complete understanding of the iodine nutritional needs of all segments of the female population in the UK.

It is worth highlighting that there has been no comprehensive investigation into the iodine levels of the population or the iodine status of women specifically in certain regions of the UK, such as Scotland and Wales. Research has been conducted regarding iodine sufficiency in various regions of the UK. For instance, Evans et al. (2014) assessed iodine sufficiency in newborns in Wales through TSH levels in blood spot samples and found no evidence of iodine deficiency in the population. Another study by Bath et al. (2016) examined iodine levels in children aged 8-10 in South-East England, Scotland, and Northern Ireland, and suggested that higher milk consumption had led to iodine repletion in this age group. Additionally, Mullan et al. (2020) investigated the iodine status of adolescent girls across the Irish islands, and the overall median UIC was found to be at the lower end of sufficiency. While some information is available regarding iodine status among teenage girls and women of childbearing age in different regions of the UK, it appears there might be a lack of detailed, region-specific studies.

The fluctuation score and its relationship with UIC

Second, we examined the UIC status using the fluctuation rating concept that we created. We grouped UIC using the fluctuation rating groups in both

gender and the Kruskal-Wallis analysis indicated that there were significant differences between fluctuation groups in females (p=0.023); although no significance was observed in males, the Mann-Whitney test showed that UIC in the male high fluctuation group was significantly higher than that in the iodine intake stable group (Table 3.6).

Additionally, the correlation between UIC and fluctuations in iodine intake (measured by the standard deviation, SD), as well as mean iodine intake across different age groups and genders was evaluated. In girls aged between 4-12 years and male adults (19-60), there is a weak correlation between UIC and fluctuations in iodine intake (SD). This was not observed in any of the other age cohorts (Figure 3.1). However, a positive correlation between mean iodine intake and UIC was observed in all gender and age groups aside from elder females, this indicating that a higher level of mean daily iodine intake correlates with higher UIC levels (Figure 3.2).

Moreover, the correlation between the mean iodine intake (µg) with the fluctuation in daily iodine intake (SD) was analysed (Figure 3.3). And this strong and positive relationship remained consistent across different age groups and between both genders. The strong and consistent correlation between mean iodine intake and its daily fluctuation inversely suggests a potential correlation between fluctuation and UIC. This underscores the complex interplay between dietary habits and iodine status, an area yet to be thoroughly explored, particularly concerning iodine intake fluctuations. Since no research has been conducted on iodine intake fluctuations, here we try to discuss the result using UIC with mean iodine intake. Our finding of a positive correlation between mean iodine intake and UIC in early age groups aligns with the research conducted by Katagiri et al. (2016), who found a significant correlation between estimated iodine intake from four-day food diaries and urinary iodine collected over 24 hours. This similarity suggests a consistent association between dietary iodine consumption and its urinary excretion, particularly in younger populations. It can be inferred that there is a more consistent dietary intake of iodine among children. This consistency could be due to a more regulated diet, or possibly due to interventions such as school

feeding programs that offer more uniform meals. As mentioned in a review by Evans and Harper (2009), standards for school food have a positive impact on both pupils' food choices and the nutritional profiles of school lunches. In contrast, adults might not experience the same level of dietary regulation, as shown in our study, especially females. Their eating patterns are often more varied and less influenced by standardized meal programs. According to Kant and Graubard (2015) and Gill and Panda (2015), traditional eating patterns observed in adults typically exhibit more variability and less adherence to consistent meal timing and patterns compared to the structured environment experienced by children. This variability could contribute to the less consistent iodine intake observed in adult and elder populations.

Additionally, it is worth noticing that in the NDNS methodology, as outlined in our chapter introduction. The spot urine sample was collected either during the distribution or collection of the four-day food diary. Given the reported eight-week interval between the distribution and collection of these diaries, there is a potential misalignment between the recorded dietary intake and the UIC measurements. This time lag is significant which means that the iodine intake recorded in the food diaries may not directly reflect the iodine status as captured by the UIC.

Furthermore, while a correlation between mean iodine intake and UIC is identified within the NDNS data, the timing of urine sample collection—either concurrent with or separate from the period covered by the food diary—may impact this relationship. In my view, it is reasonable to hypothesize that the weak correlation observed between the SD of iodine intake and UIC may be weakened by the timing of urine sample collection relative to dietary recording. This hypothesis is supported by findings from Katagiri et al. (2016) study, they further specified that with a 4-day diet record (non-consecutive) and 24-hour urine collection, if the urine sample was collected with the same time of the food record, the correlation was stronger ($r=0.37$, $p=0.005$) compared to participants that collected the urine sample either before($r=0.31$; $p=0.01$) or after ($r=0.23$; $p=0.001$) food records. Such discrepancies underscore the importance of aligning the timing of urine collection with the

dietary recording period to accurately reflect the immediate impact of dietary iodine intake on UIC.

The approach of spot urine sample used in NDNS

As mentioned in previous section, the NDNS approach of collecting only one spot urine sample from each participant, starting at the age of four, presents limitations for accurately describing the prevalence of iodine status due to its snapshot nature. As mentioned in the general introduction 1.2.6, this methodological constraint could lead to misrepresentation of the actual iodine status across different population groups, as individual iodine excretion can vary significantly throughout the day due to dietary intake and other factors (Zimmermann, 2008, Zimmermann, 2009). Despite these limitations, the use of spot urine samples in large-scale studies like the NDNS is often justified based on practical considerations such as cost, participant compliance, and logistical feasibility. However, the use of spot urine samples for estimating population iodine intake has been questioned because these measures may not accurately predict 24-hour urine iodine excretion (Ji et al., 2015). It would be preferable to obtain multiple urine samples from each participant to account for the variation that may arise within individuals when determining intra-individual iodine concentration distribution (König et al., 2011).

Johner et al. (2010) reported that high fluid intake, indicated by the volume of 24-hour urine, is significantly associated with increased daily iodine excretion, particularly in adolescents. This suggests that urine volume should be acknowledged as a potential confounder in studies that utilise urinary iodine excretion as a biomarker. Additionally, as mentioned in the general introduction 1.2.6, variations in urinary volume can be compensated for by adjusting the iodine concentration relative to creatinine(Vejbjerg et al., 2009). It relies on constant daily urinary creatinine excretion by healthy individuals. Although urinary creatinine excretion may be affected by protein intake, sex, and age (Boeniger et al., 1993, Bourdoux, 1998), the use of creatinine to adjust UIC measurements is beneficial and common(Garçon et al., 2007, Montenegro-Bethancourt et al., 2015, Ji et al., 2020). It accounts for variations in urine dilution, offering a more standardised method to evaluate iodine

status. However, it is regrettable that, in the methodology of the NDNS, urinary creatinine was not included in the collected data, although blood creatinine levels were recorded for years 9 and 10. The failure to include urinary creatinine in the analysis hindered the ability to comprehend the iodine status of the UK population with greater precision, as it would have supplied a more detailed perspective.

Moreover, the timing of the urine sample collection is crucial, as noted by Zimmermann (2009), urinary iodine excretion levels can quickly reflect short-term dietary intakes. The study done by Als et al. (2000) investigated the circadian rhythm of UIC and found that UIC levels are lowest between 8-11 am and increase progressively between 12 pm and 12 am. This indicated that morning spot urine samples might be the most appropriate for reflecting daily iodine status. However, in NDNS no exact time of the day was recorded as to when the spot urine sample was taken, this potential misalignment could lead to an underestimation or overestimation of the true iodine intake, impacting the accuracy of the correlation between dietary records and UIC levels.

Iodine-rich Food contributions

Milk and dairy products significantly contribute to dietary iodine intake, accounting for up to 50% of the total iodine consumption among both male and female children, according to Tables 3.9 and 3.10 of our analysis. Notably, semi-skimmed milk is the primary source among these dairy products, constituting 21.41% and 21.85% of iodine intake for males and females aged 4-12 years, respectively. In contrast, adult males (19-60 years old) have the lowest rate of dairy-derived iodine intake at 34.28%, as outlined in Table 3.13. These observations align with findings from previous Western population studies, such as those by Dahl et al. (2004), van der Reijden et al. (2017b) and Bath and Rayman (2020), underscoring the predominant role of milk and dairy products in contributing to iodine intake.

It is also observed in our results that fish- and fish- related products plays a key role in affecting fluctuation of iodine intake in all age groups and genders possibly due to the high iodine content fish and fish related products.

Our results suggest that participants in the high iodine fluctuation group (fluctuation rating at 5) had significantly higher iodine intake from fish and fish products during the food recording period compared to participants who had more steady iodine intake (fluctuation rating at 1) between recorded daily intakes, across all age and gender groups. This difference was particularly notable among older participants, where the percentage of iodine from total fish intake in females with a high fluctuation score was 40.45%, and in elder males with a high fluctuation score, it was 25.36%. This is in stark contrast to participants from the same age cohort with stable iodine intake (fluctuation rating at 1), where the percentages were significantly lower, at 3.27% for females and 2.92% for males, respectively.

The NHS recommendation for consuming two portions of fish per week, including one oily fish, is aimed at promoting a healthy diet. However, the actual contribution of fish to iodine intake in the UK appears to be less significant than previously assumed.

Wenlock et al. (1982) indicated that fish and fish products, despite being rich in iodine, contributed only approximately 5% of the total iodine intake in their sample. This suggests that other dietary sources, particularly dairy products, may play a more dominant role in the typical UK diet. In a more recent study, Bouga et al. (2018) reported that fish consumption remains low in the UK, contributing only approximately 16% to the recommended adult iodine intake. This indicates a potential public health issue as iodine is essential for thyroid function and overall health. Low fish consumption may lead to inadequate iodine intake, particularly if other iodine-rich foods are not consumed in sufficient quantities. Sprague et al. (2021) found that seafood contributes only 9.4–18.0% to the UK's reference nutrient intake of iodine. However, their results were only on average for all participants separated by gender and age groups.

In our study, we observed that among individuals with more stable iodine intake (fluctuation rating at 1), fish and fish-related products contributed less to the total iodine intake level. This finding is critical, as it suggests that even among individuals who manage to maintain a consistent iodine intake, fish do not constitute a primary source., in which milk and dairy products are the predominant sources. This might reflect dietary habits, personal preferences, availability, and cultural preferences affecting fish consumption in the UK.

However, the fluctuation of iodine intake as a commonly recurring eating habit is challenging to assess solely based on short-term dietary records, such as the 4-day food diary from the NDNS. This variability in iodine intake highlights the importance of considering long-term dietary patterns, and the need for more comprehensive assessments to understand the full scope of iodine intake fluctuations in the UK population.

Limitations

One notable limitation of our study is the reliance on a single-spot urine sample for UIC results. As the WHO (2013) has already acknowledged, UIC levels can vary significantly within the same day, influenced by the consumption of iodine-rich foods and the timing of urine sample collection. Therefore, a 24-hour urine sample may offer a more accurate measure for monitoring iodine status and explaining the extreme cases observed during UIC assessment. However, collecting 24-hour urine samples, or even single-spot samples, may be met with resistance from participants.

Furthermore, the accuracy of the four-day food diary results from the NDNS relies heavily on participant honesty and reporting precision. Underreporting is a common issue, particularly among overweight and obese individuals, and women are more likely to do so than men (Macdiarmid and Blundell, 1998). A study by Murakami et al. (2008) with young (18-22) Japanese females indicated that differential underreporting of intake in terms of absolute amounts was linked to BMI, whereas underreporting of intake in terms of adjusted energy consumption was not. These factors must be considered when interpreting results from self-reported food diaries like the NDNS.

Enhancing participant training about portion size and adherence could mitigate some reporting inaccuracies.

Additionally, our analysis did not account for iodine supplementation. This oversight means that individuals who consume iodine supplements or seaweed—which was only categorized under 'vegetable not raw' and 'miscellaneous', as mentioned in Section 3.3.6, —may have been inaccurately represented in terms of iodine intake. This could particularly affect the assessment of those who depend heavily on such sources for their iodine intake, leading to potential public health concerns related to thyroid function and risks associated with excessive iodine intake.

Considering these limitations, future research should aim to capture a more comprehensive picture of habitual iodine intake patterns. We propose two approaches: conducting a four-day food diary monthly for at least 10 months to a full year, ideally with concurrent spot or 24-hour urine samples for UIC assessments; or a detailed 10-week food diary tracking with weekly urine samples. These methods would provide a clearer understanding of the fluctuations in iodine intake(Katagiri et al., 2015).

In addition to human studies, animal trials and/or cell studies can provide a controlled environment for examining the direct impact of fluctuations in iodine intake on thyroid function and general health. Trials can be conducted to manipulate iodine levels precisely and observe the resulting physiological and metabolic responses. Considering the ethical implications and relevance of the findings to human physiology, animal models and/or cell studies can provide valuable insights that complement human data and help clarify the underlying mechanisms of iodine intake fluctuations.

Chapter 4 Exploring the Impact of Iodine Fluctuations: In Vitro Studies on Thyroid Hormone Production and Cellular Stress Response

4.1 Introduction

Iodine intake often follows an irregular pattern, which is a common occurrence in our daily lives as shown in our previous chapter. The thyroid gland possesses remarkable adaptability as well as the HPT axis as a whole, which plays a critical role in ensuring TRH-TSH-TH homeostasis However, the irregularity of iodine intake fluctuation poses challenges in monitoring its potential health implications. Despite the importance of iodine in thyroid function, there is limited understanding of the impact of fluctuations in iodine levels on thyroid cells. Numerous studies have explored the impact of iodine deficiency (Krajčovičová-Kudláčková et al., 2003, Jiang et al., 2019, Lisco et al., 2023) and excess (Uberti, 2001, Teng et al., 2006b, Luo et al., 2014) on overall thyroid function and health. There is some limited research examining the effects of fairly long-term fluctuation at the public health level, mainly on seasonal changes in UIC due to differences in iodine content in milk(Soriguer et al., 2011, van der Reijden et al., 2017b, Coneyworth et al., 2020), but nothing on short term “normal” fluctuations at the cellular level. Therefore, we chose to explore the mechanism by which iodine fluctuations impact thyroid cell function using a thyroid cell culture model.

Iodine Transport, Thyroid Hormone Biosynthesis, and Regulatory Mechanisms

The transport of iodine into the thyroid cells and incorporation of iodine into thyroid hormones involve relevant genes and pathways, as depicted in the general introduction section 1.3.2, with the visual presentation of Figure 1.7. Here, we present a simplified representation as shown in Figure 4.1.

Iodine enters the circulation mainly as inorganic iodide, which is transported into the thyroid cells through the sodium iodide symporter (NIS). NIS, also known as Solute Carrier 5A (SLC5A), is a member of a family of transporters that depends on an electrochemical sodium gradient to permit the active transport of iodide into thyroid follicular cells, an initial and essential step in thyroid hormone synthesis. In addition to the thyroid, active transport of iodide has also been observed in other organs, such as the lacrimal sac and nasolacrimal duct, salivary glands, choroid plexus, stomach, small intestine,

and lactating breast (Portulano et al., 2014). NIS is located in the basolateral membrane of thyroid follicular cells, and the sodium gradient produced by Na/K-ATPase is necessary for NIS function (Dai et al., 1996, Kopp et al., 2015).

To regulate the expression and function of NIS, TSH activates the cyclic adenosine monophosphate (cAMP) pathway to stimulate NIS activity in thyroid follicular cells through THSR (Riesco-Eizaguirre and Santisteban, 2006, Huang et al., 2011, Braverman and Cooper, 2013). Both *in vivo* and *in vitro* studies show that TSH up-regulates the mRNA and protein expression of NIS (Levy et al., 1997, Kogai et al., 1997, Eng et al., 2001).

TSH is the primary regulator of NIS expression within the thyroid gland. Activation of TSH starts with its interaction with the TSHR, which, through the Gs-protein, stimulates adenylyl cyclase. This results in the accumulation of cAMP in thyroid cells. The increase in cAMP levels activates several thyroid transcription factors (TTFs), including TTF1 (also known as NK2 homeobox 1 or NKX2-1), TTF2 (also known as forkhead box protein E1 or FOXE1), paired box gene 8 (PAX8), and hematopoietically-expressed homeobox protein (HHEX). Among these, TTF1 and PAX8 are pivotal, as they regulate numerous downstream genes, including HHEX and TTF2 (Jing and Zhang, 2022). Furthermore, the DNA binding sites of TTF1 and PAX8 often overlap in the promoters of their target genes, facilitating coordinated transcriptional regulation (López-Márquez et al., 2021).

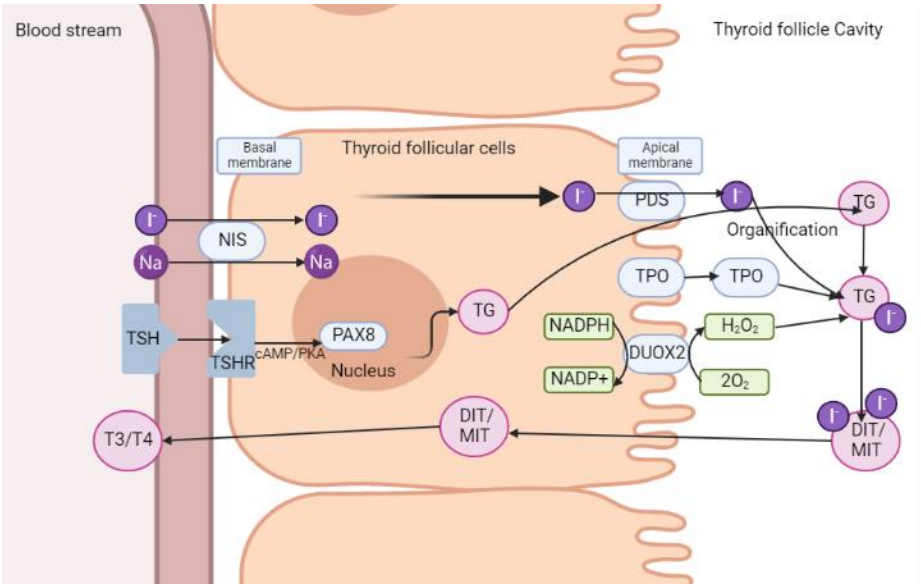


Figure 4. 1 Reproduced from the Werner & Ingbar's The Thyroid: A Fundamental & Clinical Text, 10th Edition Section1.Chapter 4B. Thyroid Hormone Synthesis. cAMP, cyclic AMP; DIT, diiodotyrosine; DUOX, dual oxidase 2; MIT, monoiodotyrosine; NIS, sodium/iodide symporter; PDS, pendrin; PKA, protein kinase A; TG, thyroglobulin; TPO, thyroid peroxidase; TSH, thyrotropin; TSHR, TSH receptor. The graph was generated with Biorender.com

In adult thyroid follicular cells, PAX8 plays a crucial role in regulating the expression of numerous genes integral to thyroid hormone production, including thyroglobulin (TG), thyroid peroxidase (TPO), and TSHR (Pasca di Magliano et al., 2000, D'Andrea et al., 2006, Jing and Zhang, 2022). PAX8 can also bind to two enhancers that are upstream NIS promoters to induce NIS transcription. Notably, studies involving Pax8−/− mice have shown that the absence of PAX8 leads to severe hypothyroidism, characterized by thyroid hypoplasia, low birth weight, developmental delays, and high mortality rates within the first few weeks post-birth (Mansouri et al., 1998).

As mentioned in general introduction, H_2O_2 is a highly reactive molecule that plays important roles in various physiological and pathological processes in living organisms. The production of H_2O_2 is predominantly carried out by reduced nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) enzymes. These enzymes utilize molecular oxygen to generate H_2O_2 or the O_2 superoxide (O_2^-), which is subsequently converted to H2O2 by the action of superoxide dismutases (SODs) (Song et al., 2007, Szanto et al.,

2019). In addition to the NADPH oxidase enzymes, mitochondria are also a major source of cellular H_2O_2 production(Loschen et al., 1974).
Iodide oxidation, organification, and coupling reactions depend on the presence of H_2O_2. The system of thyroid follicular cell H_2O_2 generation is located at the apical membrane and produces H_2O_2 by oxidising NADPH with an NADPH oxidase. To mirror the dual oxidase and peroxidase abilities, the proteins were named DUOX1 and DUOX2, both of which are calcium-activated to generate H_2O_2 from O_2 (Brandes et al., 2014). DUOX2 and TPO are two primary enzymes involved in the redox phase of thyroid hormone production. TPO catalyzes the oxidative iodination reaction and plays an important role in thyroid hormone production. DUOX2 provides H_2O_2 that TPO needs as an electron acceptor in these processes, which is catalysed by TPO and involves the iodination of TG tyrosine residues and subsequent iodotyrosine chain coupling(Szanto et al., 2019). The process mediated by TPO is complex; H_2O_2 (ROS) generated during the process is crucial for the organification process but also poses a risk of oxidative damage to thyroid cells if not properly regulated or neutralised (McLachlan and Rapoport, 1992, Godlewska and Banga, 2019).

Under iodine deficiency conditions, the scenario becomes even more threatening. The research done by Ehrenshaft and Mason (2006) indicate that TPO generates radical adducts in the presence of H_2O_2, the presence of Iodide strongly suppresses the H_2O_2-generated production of TPO radical adducts and protects the enzyme from loss of enzyme activity. This untargeted radical production can exacerbate oxidative stress and contribute to the pathogenesis of thyroid disorders (Ehrenshaft and Mason, 2006). The relationship between TPO activity, iodine availability, and ROS production emphasises the delicate balance that needs to be maintained for optimal thyroid function. This highlights the potential for iodine deficiency to disturb this balance, causing thyroid pathology and autoimmunity(Taurog et al., 1996).

The endoplasmic reticulum (ER) is a crucial organelle responsible for maintaining proper folding, trafficking, and degradation of newly synthesised

proteins through a process known as ER quality control (Guerriero and Brodsky, 2012). This process is essential to ensure the integrity of cellular protein production. In the thyroid follicles, thyrocytes synthesise a precursor protein TG in the ER, which does not contain iodine, but is the key component of TH synthesis. Thyrocytes have a high demand for secretory proteins to support thyroid hormone synthesis, making them particularly vulnerable to environmental factors that induce ER stress. Studies have shown that ER stress can disrupt the proper glycosylation of TG, impairing its function as a major secretory protein and leading to its retention in the ER (Leonardi et al., 2002). This highlights the critical role of the ER in maintaining protein homeostasis and the potential consequences of ER stress on cellular function. A study conducted by Wen et al. (2017) claimed that ER stress may cause downregulation of genes involved in thyroid hormone synthesis, including NIS, TG, and TPO, through impaired TSH/TSHR signalling.

The protein glucose-regulated protein 78 (GRP78), serves as a surveillance system for ER stress. In mammals, the apoptotic pathway triggered by ER stress plays a crucial role in cell death (Kim et al., 2008). Various disturbances, including oxidative stress, can disrupt proper protein folding and lead to the build-up of misfolded or unfolded proteins in the ER. This activates a well-conserved response, known as the “unfolded protein response” (UPR), which aims to restore homeostasis and normal ER function.

Autophagy is often activated as a protective mechanism in response to ER stress, serving to eliminate misfolded or damaged proteins and link cellular responses between the ER and autophagic pathways(Rashid et al., 2015). Autophagy is a catabolic process that is crucial for maintaining cellular homeostasis and adapting to changing environmental conditions (Rubinsztein et al., 2005). During this process, double-membrane structures known as autophagosomes are formed, which engulf cytoplasmic components such as cytosolic proteins and organelles. This process is regulated by a complex signalling network and involves the recruitment of various proteins, including LC3 (microtubule-associated protein 1A/1B-light chain 3). Upon the initiation of autophagy, the cytosolic form of LC3 (LC3-I) is converted to its lipidated

form, LC3-phosphatidylethanolamine conjugate (LC3-II), through the action of Atg7 and Atg3 enzymes (Tanida et al., 2008, Tanida et al., 2005). This process allows the incorporation of LC3-II into the autophagosomal membrane, where it plays a key role in autophagosome formation and maturation. The detection of LC3-II is widely used as a marker for autophagy and is commonly assessed using techniques such as western blotting and immunofluorescence microscopy. Overall, the recruitment of LC3 to autophagosomes is a critical step in the autophagic process and serves as a key indicator of autophagy activity.

A study conducted by Zhang et al. (2015) investigated the levels of LC3-II in different thyroid diseases and revealed that in nodular goitre and Hashimoto thyroiditis tissues, there was higher expression of LC3-II protein compared to normal thyroid tissue. Furthermore, the results also showed a lower level of autophagic activity in papillary thyroid cancer samples. There was no significant correlation between patient sex and LC3-II levels.

Correct cellular function depends on balanced cellular redox homeostasis; consequently, cells must maintain a proper balance between the oxidative and antioxidant status. This is important in thyroid cells for thyroid hormone production, because production requires ROS generation by the NADPH oxidase family external to the mitochondrial respiratory chain. Any disruption in this delicate balance can lead to detrimental effects on thyroid hormone production and ultimately impair cellular function.

Assessment of the impact of iodine fluctuation on thyroid cells in vitro.

There has been little or no assessment of the impact of fluctuating iodide exposure using *in vitro* systems. The majority of studies to date have explored other aspects of thyroid cell biology and have employed a range of cell lines, including rat thyroid cells, human thyroid cells, and thyroid cancer cell models, such as PTC and FTC (Saiselet et al., 2012). For the current study we specifically required a normal (non-diseased) thyroid cell line, thereby ruling out the use of thyroid cancer cell models and the commonly employed rat derived FRTL-5 (Grollman et al., 1986, Peter et al., 1991) and PCCL3 (Feliciello et al., 2000, Kimura et al., 2001); Nthy-ori 3-1 is a widely used

(Piotrowska et al., 2011, Kurashige et al., 2020b) cell which was developed from normal human thyroid.

Both rat thyroid cell lines were spontaneously immortalised and their growth was entirely dependent on TSH levels. However, their reliance on a high concentration of six hormones (6H) for viability has raised concerns about the practicality and cost-effectiveness of their use in experiments. In addition, they have been reported to suffer from instability (Kimura et al., 2001). The research done by Peter et al. (1991) suggested that, in a comparable environment, the proliferation rate of FRTL-5 was higher than that of the normal follicular epithelium in the thyroids of the host animal.

In contrast, Nthy-ori 3-1 is derived from a normal human adult thyroid and retains epithelial morphology. In addition, there was a report from Shi et al. (2016) on successfully using Nthy-ori 3-1 to create a transwell model, highlighting its potential for use in *in vitro* studies. Notably, in their study, this cell line was shown to maintain key features of normal thyroid epithelial cells, such as the ability to secrete thyroid hormones and respond to thyroid stimulating hormone (TSH) signalling. Overall, the unique properties of Nthy-ori 3-1 as a thyroid cell line option, make it a valuable tool for investigating thyroid biology.

The selected thyroid cell line will served as the foundation for constructing an in vitro model and devising an iodine fluctuation model, with the intention of utilizing a transwell insert to enable the study of the thyroid hormone synthesis pathway and its response to fluctuation concentration of iodine.

Because reactive oxidative species play a crucial role in thyroid hormone synthesis (facilitating iodide oxidation, organification, and coupling reactions), maintaining a balance between oxidative and antioxidant status is crucial. Any disturbance in this balance can negatively affect thyroid hormone production and impair cell function. Therefore, we aimed to assess the ability of thyroid

cells to generate ROS and defend against high levels of extracellular oxidative damage (specifically H_2O_2). We additionally wanted to determine whether fluctuations in iodine supply might potentiate the cellular responsiveness to ROS generation or establish if this might play a causative role.

We first established a reliable experimental system using the Nthy-ori 3-1 human thyroid follicular cell line as a model by assessing the effectiveness of Transwell inserts in comparison with traditional static cultures. Once the model was established, we investigated the impact of fluctuating iodine levels on the expression of genes related to the production of thyroid hormones, generation of reactive oxygen species (ROS), and the ability of the cells to withstand external oxidative stress in the context of the iodine fluctuation model.

4.2 Method

4.2.1 Cell culture

In this chapter Nthy-ori 3-1 human thyroid epithelial cell line was used. are described in detail in Section 2.1.5.

4.2.2 Composition of the solution and treatment condition

The majority of thyroid cell studies previously used 100mUI/L (Lyu et al., 2018); 1mUI/mL TSH to treat cells, although these studies either used the rat thyroid cell lines PCCL3 and FRTL5, or primary thyroid cells. Additionally, a study using Nthy-ori 3-1 cell line to conduct a transwell thyroid follicule model was using 1mUI/L

The TSH concentration used in this study was slightly higher than the standard human TSH level (0.5-4.5mIU/L), 10mIU/L.

The iodine used in this chapter was in the form of iodide (I^-) sourced from Potassium Iodide (KI, ACS ACROS Organics). It is a widely used source, as shown in many related studies, and as a source of iodine that rapidly occupies thyroid cells to prevent thyroid exposure to radioactive iodine(I-131)(Reiners and Schneider, 2013).

4.2.3 Determination of iodine tolerance in thyroid cell

As we have not previously worked with Nthy-ori 3-1, a human thyroid follicular cell line, it is necessary to evaluate the iodine tolerance level of the target thyrocyte.

To test the possibility of iodine tolerance in the thyroid cell line Nthy-ori 3-1, Nthy-ori 3-1 cell were cultured in 12 well plates and treated with different concentrations of Potassium Iodide (KI), ranging up to 2000 mg/L (1000 mg/L of iodide for the first test trial) for 24 and 48 h. Afterwards, the cell viability was tested using CCK-8; the experimental steps for the CCK-8 were described in section 2.2.2.

4.2.4 The application of the Transwell model to form an in vitro thyroid follicle unit.

Transwell insert chambers with a permeable polycarbonate membrane (Transwell multiple well plate inserts with 0.4um pore size; 6 well 3412; 12 well 3401, Corning) were used to build the transwell model.

First, the membrane of the insert chamber was coated with collagen (type I C8919, Sigma-Aldrich) and 10μg/cm2 of collagen I was used for coating. The collagen I-coated plate with the insert chamber was left in the hood with the plate lid off, and it was exposed to UV light overnight within the hood for sterilisation. The following day, any extra liquid within the plate was removed. After that, the cells were seeded into the insert chamber (Figure 5.2), and the plate was then placed in a 37 °C incubator for at least one week to ensure full confluence in the insert chamber.

Subsequently, the media within the upper and bottom chambers of the Transwell unit were changed, as shown in Figure 5. The bottom chamber was changed to culture medium A: growth media with 10mIU/L of TSH and 500μg/L iodide. The medium in the upper chamber represents the inside of the thyroid follicle; thus, culture medium B was RPMI 1640 with 1% pen strep and no FBS. The cells in the Transwell chamber were incubated within the

treatment for another 48 h before media collection. To test whether the transwell model was successful, as the in vitro thyroid follicle model could generate fT4 within the culture media, the Human Thyroxine ELISA Kit (ab108662, Abcam) was used to assess any fT4 within the culture media from the upper chamber. The detailed method of the ELISA is described in the Methods section.

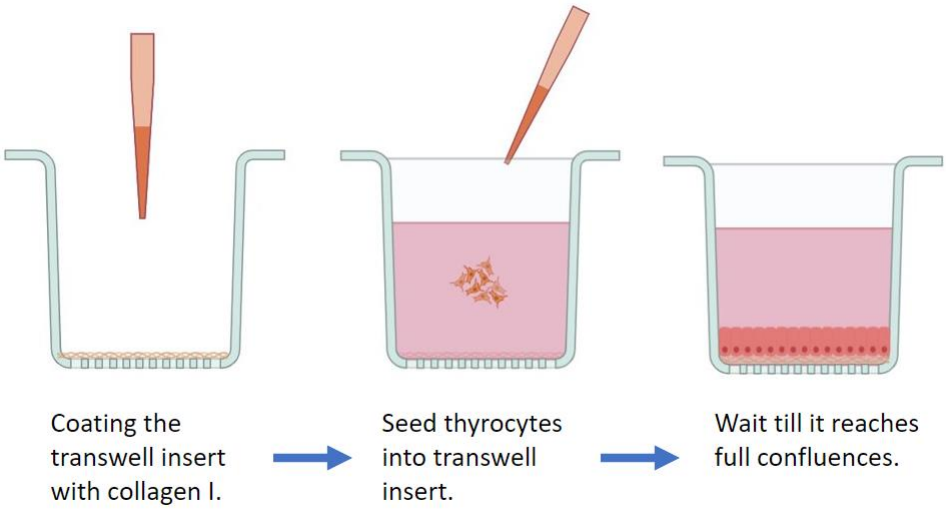


Figure 4. 2 **Transwell protocol**

Steps of making the Transwell model are shown with graphical indications. Figure was generated with Biorender.com

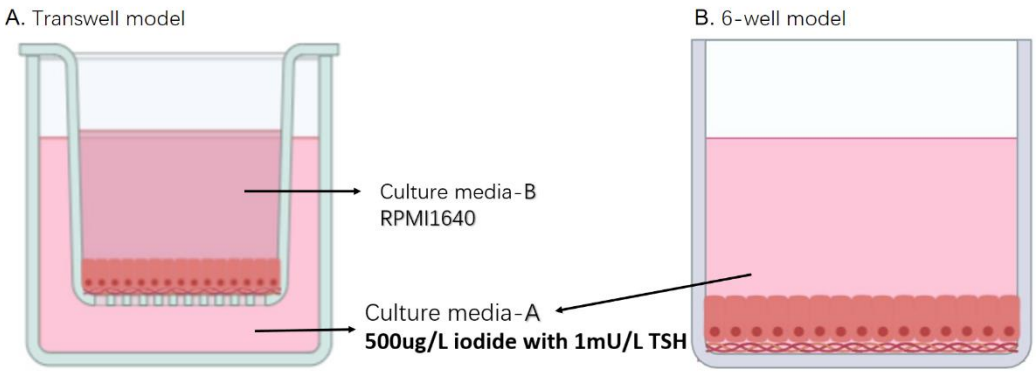


Figure 4. 3 **Transwell model and 6 well model as comparison indication**

Graphical indication of the treatment for A) cell seeded into the transwell insert chamber; B) cell seeded into 6 well plates. Figure was generated with Biorender.com

4.2.5 The design of the Fluctuation model

In this chapter, we developed a model to examine the fluctuation of iodine intake in vitro, which is similar to the situation observed in the NDNS study.

The model we developed use the term "fluctuation cycle" generally refers to a pattern of iodine intake that alternates between periods of reduced intake and increased intake. In this particular experiment, the intake of iodine was deliberately reduced to zero during the reduced intake phases, while it was increased to levels of either 500 μg/L or 10, 000 μg/L of iodide in the culture media during the increased intake phases. For the purposes of this study, a single cycle was defined as consisting of one reduced-intake phase (no iodine treatment), one increased-intake phase (iodine treatment). These phases were referred to as the "down" phase and the "up" phase of the fluctuation cycle, respectively. This model further investigated iodine fluctuations in thyroid cells with and without TSH stimulation. To achieve this, the fluctuation cycle also included the culture environment with continuous TSH stimulation (10mIU/L) as a baseline with or without a range of iodine fluctuations. In this study, all experiments were conducted after the cells had reached the third flutuation cycle.

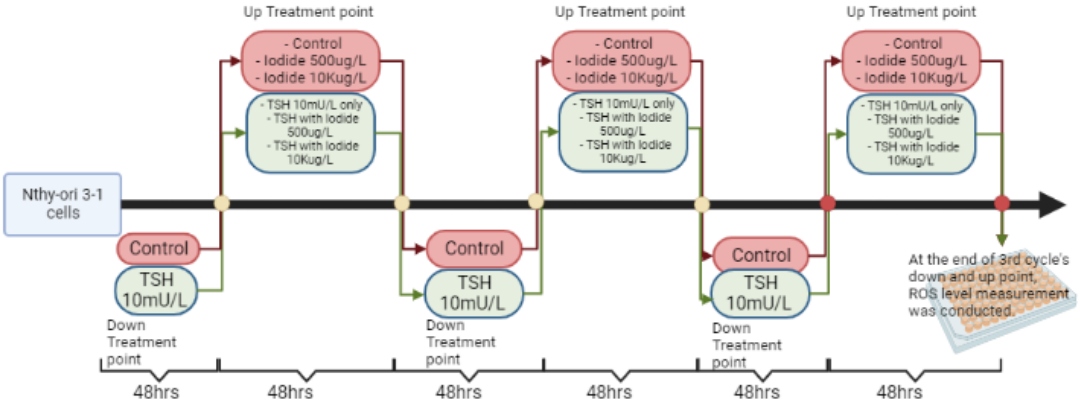


Figure 4. 4 **Timeline of treatment groups for fluctuation model.**

Nthy-ori 3-1 cells were treated with two different fluctuation sets; the difference was with or without 10mIU/L TSH within the culture media. The cell then fluctuated between the condition with or without iodide in the culture media (500µg/L or 10,000 µg/L of iodide); both stages last 48 hours, one down treatment stage and one up treatment stage count as one cycle. Finally, in the third cycle, the ROS level measurement was conducted after either the down or up treatment stage (as shown in the red dot on the figure timeline). The graph was generated with Biorender.com

Table 4. 1 Solution and treatment condition of fluctuation model

		Solution					
Treatment Groups		Control	1	2	3	4	5
Composition	Iodine-Down Phase	RPMI-1640	RPMI-1640	RPMI-1640	RPMI-1640 TSH 10mIU/L	RPMI-1640 TSH 10mIU/L	RPMI-1640 TSH 10mIU/L
	Iodine-Up Phase	RPMI-1640	RPMI-1640 Iodide 500µg/L	RPMI-1640 10,000µg/L	RPMI-1640 TSH 10mIU/L	RPMI-1640 TSH 10mIU/L Iodide 500µg/L	RPMI-1640 TSH 10mIU/L Iodide 10,000µg/L

4.2.6 fluctuation sample collection

Following the completion of the second cycle of fluctuation, as depicted in Figure 4.3, the cells were subjected to trypsinization and re-seeding into six-well plates, which were meticulously labeled to ensure accurate group assignment. After allowing the cells to settle into the plate for an hour, the third cycle of fluctuation was initiated according to the designated group.

At the end of the down and up phases of the third cycle, cell samples were collected for either cDNA extraction or protein extraction. At each sample collection point, the medium was removed, and the samples were washed twice with ice-cold PBS before collection.

For cDNA extraction, 300μL of RNase-free PBS was used to collect sample cells. Details of the collection, primers, and the rest of the experimental steps regarding qPCR are described in Section 2.5.

For protein extraction, 150μL of lysis buffer was used. The collection and rest of the experimental steps for western blotting are described in Chapter 2.6.

Antibodies used in this chapter: Rabbit Anti-Sodium Iodide Symporter antibody (ab83816, Abcam); Rabbit Anti-LC3B antibody (EPR18709, Abcam); Rabbit GRP78 Polyclonal Antibody (#PA5-34941); Rabbit Anti-Actin (A2066, Sigma- Aldrich); anti-rabbit IgG and HRP-linked antibody (#7074, CST).

All antibodies were diluted according to the protocol suggested, with 5% skimmed milk in 1x TBS-T, and the secondary antibody was diluted 1:10000 with 5% skimmed milk in 1x TBS-T.

4.2.7 Assessment of TSH-stimulated cellular ROS rate and cell sensitivity to exogenous ROS in the Nthy-ori 3-1 cell line.

To evaluate the levels of cellular ROS induced by TSH and the sensitivity of the Nthy-ori 3-1 cell line to external ROS under various circumstances ROS assays were performed.

As mentioned in the section 2.3 the detail of the assay for ROS level measurement, the ROS level of the treatment was measured inside the 96 well plate. The fluorescent dye, CM-H2DCFDA, was used. The measurements for each treatment group and oxidative stress level were performed in triplicate, with the same treatment group being repeated on the same plate three times. Different plate was used for conducting different H_2O_2 levels as 0, 0.5 and 2mM.

Reacting to the fluctuation model.

As mentioned in Section 4.2.5, after the second fluctuation cycle was completed, Nthy-ori 3-1 cell were trypsinised and re-seeded into 96 well plates (50,000 cells per well). After allowing the cells to settle in the plate for an hour, it was changed to a different treatment. The treatment group is shown in Table 4.1, and the control group was also included in the plate.

The incubation duration was 48 hours for both up and down phase, and the ROS level measurement was conducted every 24 hours.

React to continuous incubation of iodine

To compare the effects of the fluctuation model, a continuous iodine incubation with TSH levels on the thyroid cell line Nthy-ori 3-1 was conducted. The cells were seeded into 96 well plates at a density of 10,000 cells per well. After leaving the 96 well plates in an incubator at 37ºC and 5% CO2 overnight, cells were treated the following day with the following treatments: Group A (Control) received no TSH and no iodide; Group B was treated with 500 µg/L iodide without TSH; Group C received 10000 µg/L iodide without TSH; Group D was treated with 10 mIU/L TSH without iodide; Group E received a combination of 10 mIU/L TSH and 500 µg/L iodide; and Group F was treated with a combination of 10 mIU/L TSH and 10000 µg/L iodide. The incubation duration was 144 h (6 days in total), and the ROS levels were measured every 24 h.

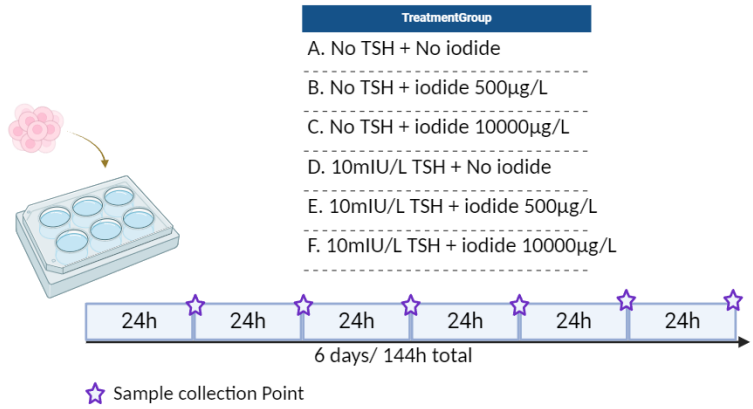


Figure 4. 5 **Timeline of treatment groups for continuous iodine incubation model.**

Nthy-ori 3-1 cells were treated with TSH (10mIU/L) alone; iodide alone (500 µg /L or 10,000 µg /L) or TSH (10mIU/L) with iodide (500 µg /L or 10,000 µg /L) for 6 days. The ROS level measurement was conducted every 24 hours as indicated with the red line on the total time.

4.2.8 Statistical Analyses

Statistics were performed using GraphPad Prism 9.3.1. All data presented in either graph or table were shown as Means ± Standard error of the mean (SEM). Analysis was specified in each experiment, either two-way or three-way ANOVA was employed, particular statistical tests performed are described in the detail of each section. and $p<0.05$ was considered as statistically significant.

The western blot data was quantified by Image Lab (V5.2.1 Bio-Rad).

4.3 Result

4.3.1 The determination of iodine tolerance level in thyroid cells

Cell viability assay using CCK-8 was performed at 24 h and 48 h with Nthy-ori 3-1 cell; they were incubated with a range of large quantities of KI to determine the iodine tolerance ability of the thyroid cells (Figure 4.6).

The first set of iodine levels (in the form of iodide) ranged from 10 mg/L to 1000 mg/L, as shown in Figure 4.6 A; there were no significant differences in cell viability levels across all tested iodide concentration groups at both 24 h and 48 h.

In order to further investigate the iodine tolerance level, an expanded iodide concentration was used to perform a second experiment (Figure 4.6 B) with the iodide levels from 100 mg/L to 2000 mg/L; assessed the cell viability at the same time points (24 and 48 hours). The results showed that there was no statistical difference in thyroid cell viability at all concentrations of iodine tested in comparison with the control group. This suggests that in the thyroid cell line, Nthy-ori 3-1, 2000 mg/L of iodide incubation for 48 h did not affect cell viability.

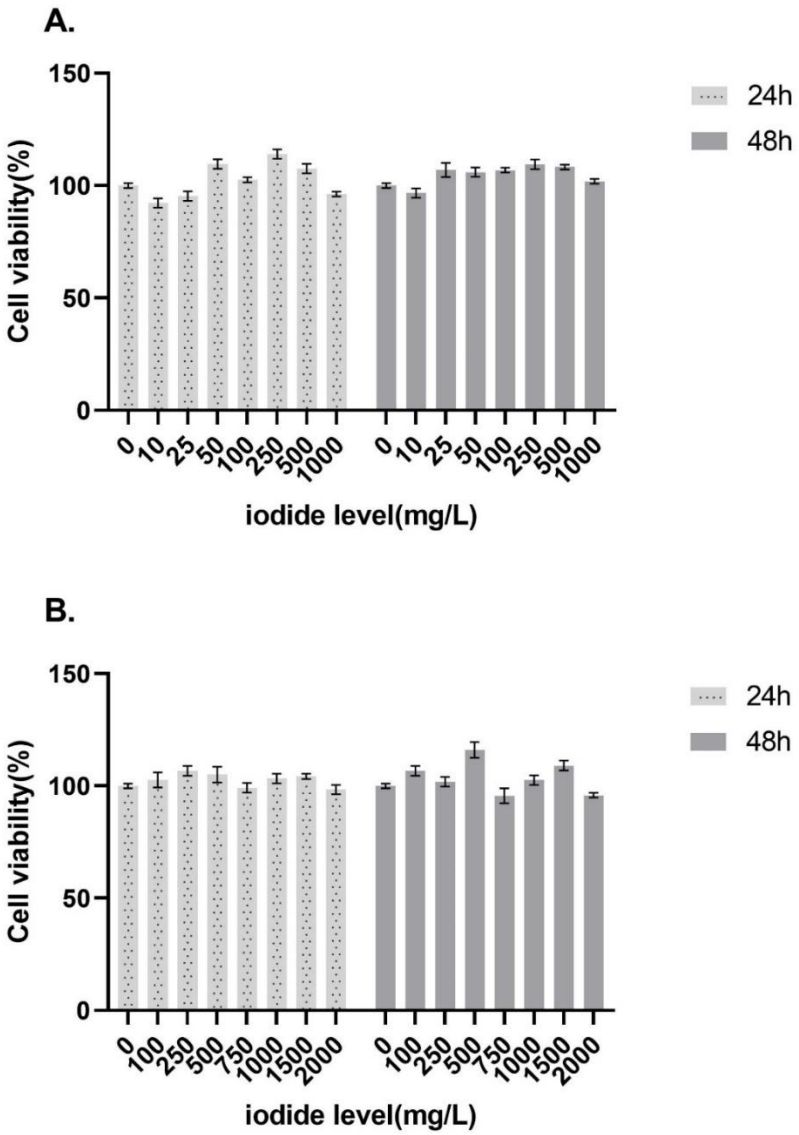


Figure 4. 6 **Cell viability of Nthy-ori 3-1 with a range of potassium iodide (KI) to measure the iodide tolarence level with the cell**

Cell viability level of human thyroid cell Nthy-ori 3-1 treated with a range of KI which contains iodide (A. Iodide concentration range from 10mg/L to 1000mg/L; B. Iodide concentration range from 100mg/L to 2000mg/L). All data were shown as mean ± SEM (n=3).

4.3.2 Impact of Iodine Fluctuations on TSH-Regulated Gene Expression in Thyroid Signalling Pathways

After constructing the fluctuation model and collecting samples, RT-qPCR analysis was conducted on samples from the third cycle of both the fluctuation down phase (48 hours of iodine deprivation) and the fluctuation up phase (48 hours of iodine incubation following 48 hours of iodine deprivation).

TSH is a pivotal regulator of THs synthesis and secretion in the thyroid gland, and its effects are facilitated by TSHR. To analyse the interactions between iodine and TSH across different fluctuation phases, two-way ANOVA was employed. The results are shown in Figure 4.7.A and B indicate that during the fluctuation down phase, there was a significant interaction between iodine levels and TSH, affecting the mRNA expression of TSHR ($p = 0.024$). Similarly, during the fluctuation up phase, a significant interaction between iodine and TSH was also detected ($p < 0.001$).

Among the TTFs activated by the TSH-TSHR pathway, as illustrated in Figure 4.7.C and D, PAX8 did not show a significant two-way interaction between iodine levels and TSH presence during the fluctuation down phase ($p = 0.249$). However, TSH presence significantly influenced PAX8 mRNA expression ($p = 0.031$). In the fluctuation up phase, a significant two-way interaction between iodine levels and TSH presence was observed, affecting PAX8 mRNA expression ($p = 0.009$)

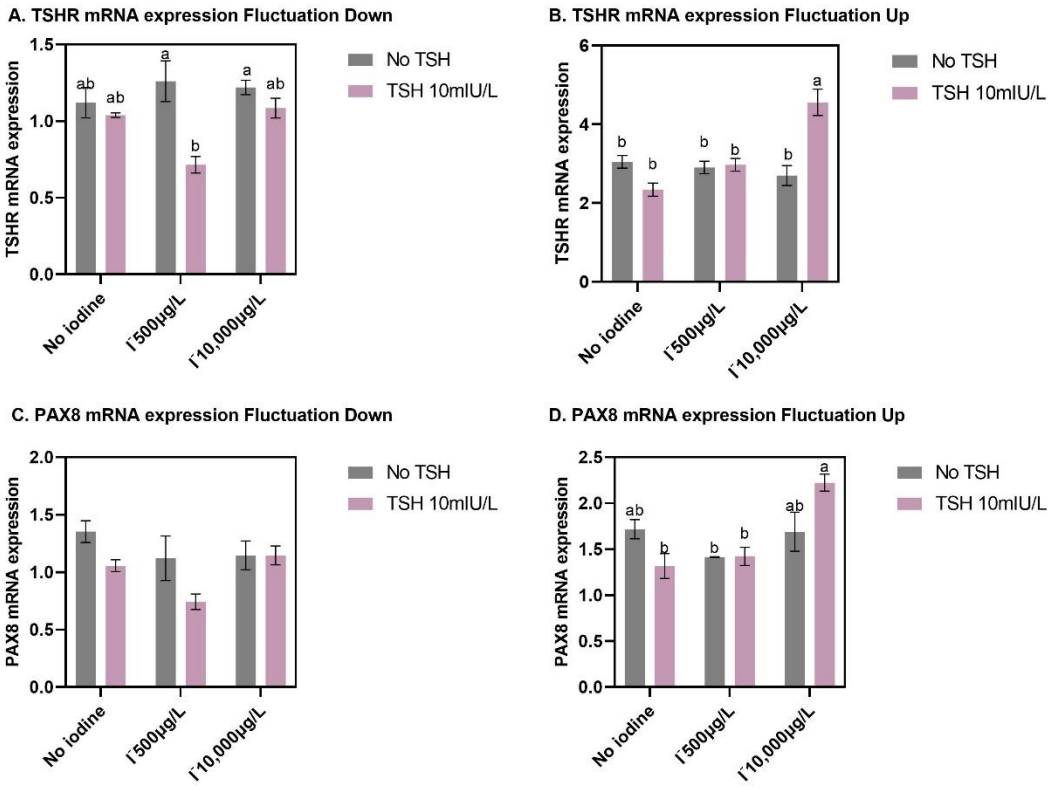


Figure 4. 7 TSHR and PAX8 expression in different phase of the fluctuation model.

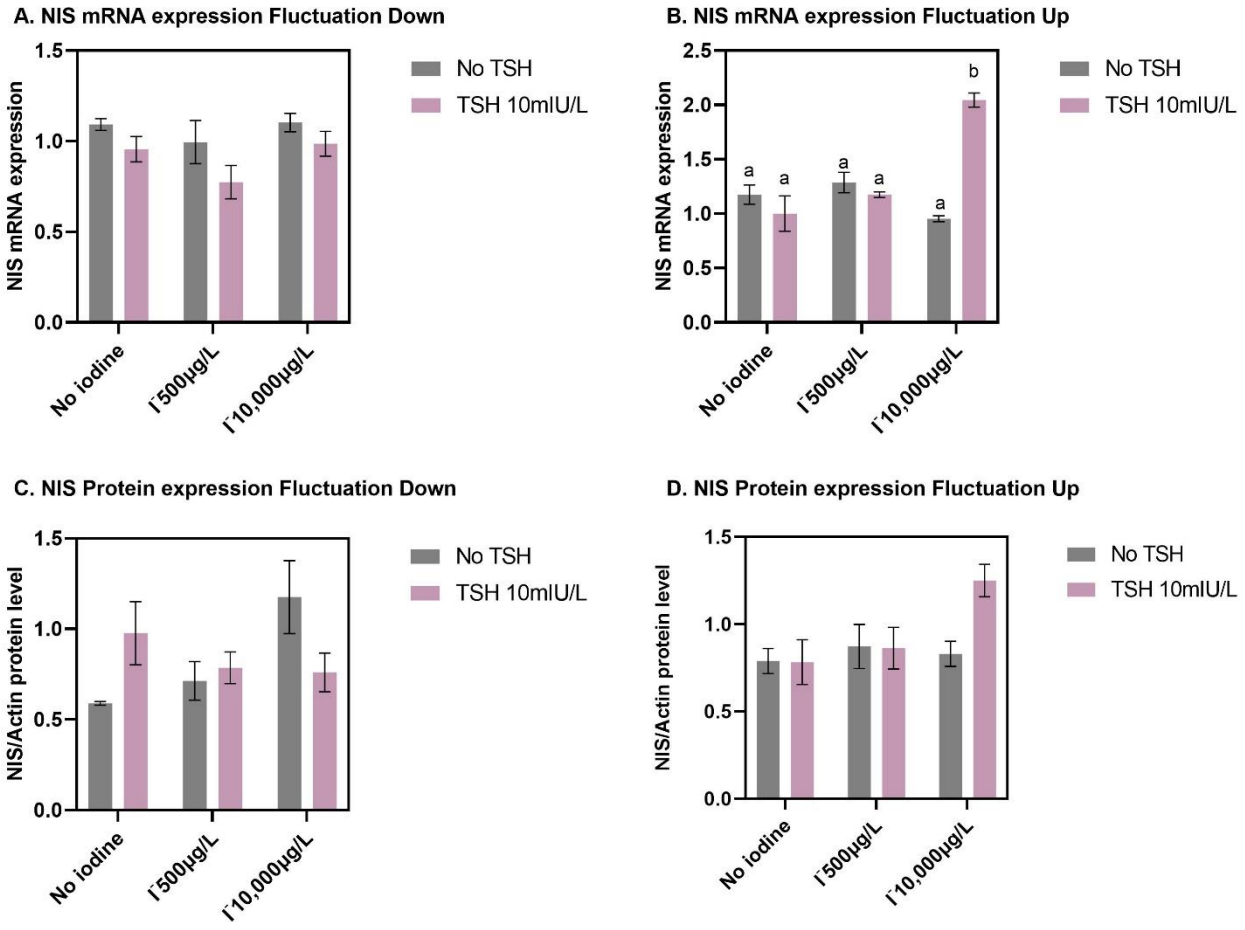
Nthy-ori 3-1 cell in fluctuation model TSHR mRNA expression in (A)fluctuation down phase; (B) fluctuation up phase; PAX8 mRNA expression in (C) fluctuation down phase; (D) fluctuation up phase. All data are expressed as mean± SEM, n=3 for each treatment group. Two-way ANOVA was used to analyse the data followed by Post hoc Bonferroni, means with the same letter are not significantly different ($p>0.05$), whereas those with different letters are significantly different ($p<0.05$).

4.3.3 Effects of Iodine Deprivation and Reintroduction on NIS mRNA and Protein Expression Mediated by TSH Interaction

NIS plays a crucial role in the thyroid hormone production pathway. During the iodine deprivation phase (Fluctuation Down), there was no significant two-way interaction between iodine level and TSH on NIS mRNA expression ($p = 0.779$), nor was there a significant effect of iodine level alone ($p = 0.12$). However, TSH levels significantly impacted NIS mRNA expression ($p = 0.028$), suggesting that TSH alters NIS mRNA synthesis in the absence of iodine. Upon reintroduction of iodine into the culture environment, a highly significant interaction between iodine and TSH levels was observed affecting NIS mRNA expression ($p < 0.001$).

In contrast, the trends in NIS protein expression were different. As shown in figure 4.8 C and D, in the Fluctuation Down phase, the interaction between iodine and TSH levels was significant ($p = 0.029$), influencing NIS protein expression. In the Fluctuation Up phase, the interaction between iodine and TSH levels was not significant ($p = 0.102$), though the abundance of NIS protein exhibited a pattern similar to that of the mRNA. Furthermore, TSH levels did not significantly impact NIS protein expression ($p = 0.14$), and the effect of iodine level was marginally non-significant ($p = 0.083$).

The significant regulation of NIS mRNA expression by TSH, contrasted with the less clear effects on NIS protein expression, suggests possible post-transcriptional regulation or delayed responses in protein expression compared to mRNA levels.


A. NIS mRNA expression Fluctuation Down
NIS mRNA expression
1.5
1.0
0.5
0.0
No TSH
TSH 10mIU/L
No iodine
I-500μg/L
I-10,000μg/L
B. NIS mRNA expression Fluctuation Up
NIS mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
a
a
a
a
a
b
No TSH
TSH 10mIU/L
No iodine
I-500μg/L
I-10,000μg/L
C. NIS Protein expression Fluctuation Down
NIS/Actin protein level
1.5
1.0
0.5
0.0
No TSH
TSH 10mIU/L
No iodine
I-500μg/L
I-10,000μg/L
D. NIS Protein expression Fluctuation Up
NIS/Actin protein level
1.5
1.0
0.5
0.0
No TSH
TSH 10mIU/L
No iodine
I-500μg/L
I-10,000μg/L

E. **Fluctuation down phase**	Control Iodine 500µg/L Iodine 10,000µg/L TSH only(10mU/L) TSH+Iodine 500µg/L TSH+Iodine 10,000µg/L
NIS (69kda)	
Actin (42kda)	
F. **Fluctuation Up phase**	Control Iodine 500µg/L Iodine 10,000µg/L TSH only(10mU/L) TSH+Iodine 500µg/L TSH+Iodine 10,000µg/L
NIS (69kda)	
Actin (42kda)	

Figure 4. 8 **Sodium/Iodide symporter (NIS) protein and mRNA expression in the two phases of the fluctuation cycle**

Nthy-ori 3-1 cell in fluctuation model NIS mRNA expression in (A)fluctuation down phase; (B) fluctuation up phase; NIS protein expression in (C) fluctuation down phase; (D) fluctuation up phase; (E) & (F) immunoblot image of NIS and Actin for the two different fluctuation phases. All data are expressed as mean± SEM, n=3 for each treatment group. Two-way ANOVA was used to analyse the data followed by Post hoc Bonferroni, means with the same letter are not significantly different ($p>0.05$), whereas those with different letters are significantly different ($p<0.05$).

4.3.4 Effects of TSH and Iodine Fluctuations on the Expression of Iodide Transport and Oxidation Genes in Thyrocytes

The genes related to iodide transport through the thyrocyte apical membrane (PDS), as well as those involved in iodide oxidation (TPO and DUOX2), were analysed in the fluctuation model.

During the iodine deprivation phase (Fluctuation Down), there was no significant interaction between TSH and iodine concentrations affecting PDS mRNA expression (p=0.118). However, iodine had a statistically significant effect on PDS mRNA expression (P =0.048). Conversely, during the iodine reintroduction phase (Fluctuation Up), a significant interaction between TSH and iodine concentrations significantly influenced PDS mRNA expression as shown in Figure 4.9 B (p=0.038).

The fluctuation model also explored the iodide oxidation catalyst TPO. In the Fluctuation Down phase, as shown in Figure 4.9 C there was no significant interaction between TSH and iodine concentration in terms of TPO mRNA expression (p=0.052). However, a statistically significant effect of TSH on TPO mRNA expression was noted (p=0.023). In the Fluctuation Up phase, a significant interaction between TSH and iodine concentration was found to significantly affect TPO mRNA expression ($p < 0.001$), with a notable increase in TPO expression in thyrocytes treated with 10 mIU/L TSH and 10,000 µg/L iodine.

Finally, we analysed the mRNA expression of DUOX2, an intracellular H_2O_2 generator crucial for iodide oxidation. In the Fluctuation Down phase, no significant interaction was observed between TSH and iodine concentrations (p=0.077), although iodine concentrations significantly affected DUOX2 mRNA expression (p=0.048). In contrast, the Fluctuation Up phase showed a significant interaction between TSH and iodine, which affected DUOX2 mRNA expression (p=0.005).

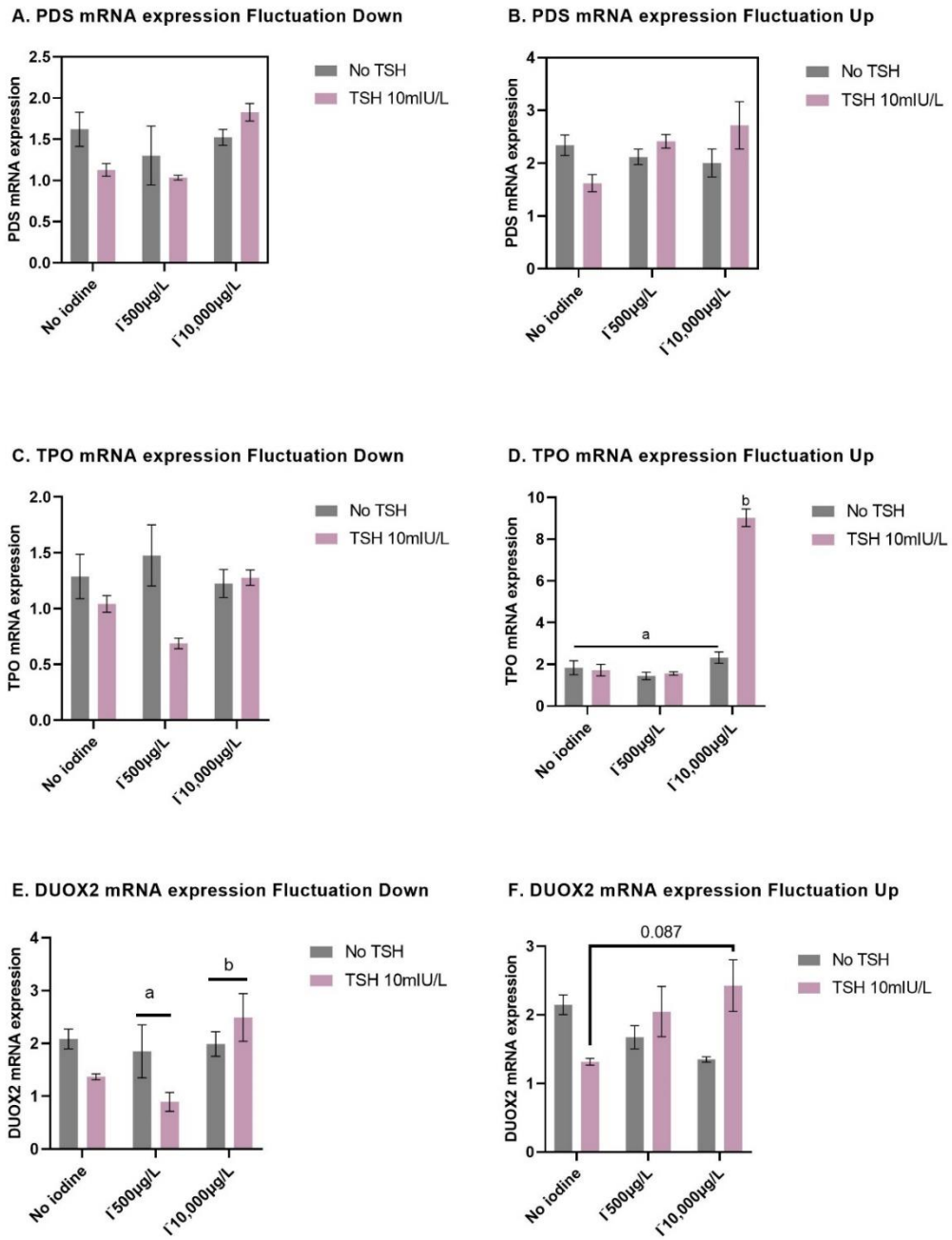


Figure 4. 9 **mRNA expression of genes related to thyroid hormone production pathway in the two phases of the fluctuation model.**

Nthy-ori 3-1 cell in fluctuation model PDS mRNA expression in (A)fluctuation down phase; (B) fluctuation up phase; TPO mRNA expression in (C) fluctuation down phase; (D) fluctuation up phase; DUOX2 mRNA expression (E) fluctuation down phase and (F) fluctuation up phase. All data are expressed as mean± SEM, n=3 for each treatment group. Two-way ANOVA was used to analyse the data followed by Post hoc Bonferroni, means with the same letter are not significantly different ($p>0.05$), whereas those with different letters are significantly different ($p<0.05$).

4.3.5 Effects of Iodine Deprivation and Reintroduction on TGF-β1 mRNA Expression in Response to TSH and Iodine Concentrations

The gene primarily involved in cell growth regulation and tissue homeostasis, TGF-β1, was examined in the fluctuating model. As demonstrated in Figure 4.10 A&B, in the Fluctuation Down phase, there was no significant interaction between TSH levels and iodine concentrations that affected TGF-β1 mRNA expression ($p = 0.106$). However, iodine alone had a statistically significant effect on TGF-β1 mRNA expression ($p = 0.022$) in the iodine deprivation phase, indicating that iodine directly influences TGF-β1 expression, independent of TSH regulation.

Iodine Reintroduction Phase (Fluctuation Up): Conversely, during this phase, a significant interaction was observed between TSH and iodine concentration, which notably influenced TGF-β1 mRNA expression ($p = 0.006$). This suggests that upon the reintroduction of iodine, the combined effect of TSH and iodine significantly affects the regulation of TGF-β1.

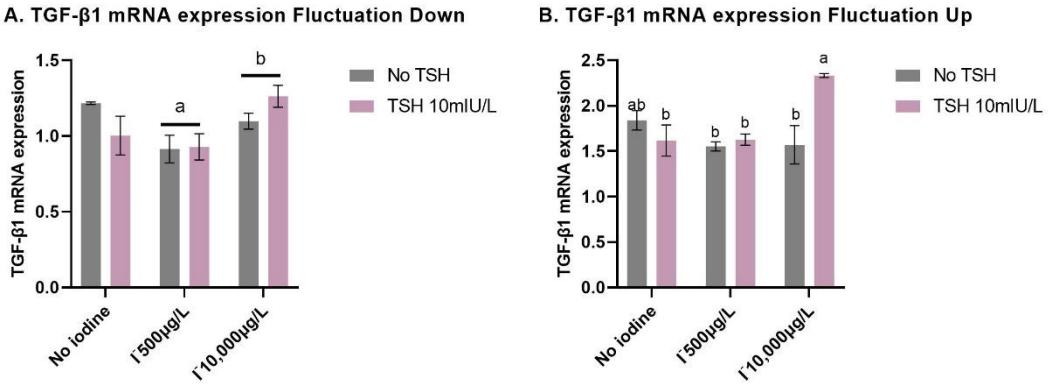


Figure 4. 10 **TGF-** β1 **mRNA expression in the two phases of the fluctuation cycle**

Nthy-ori 3-1 cell in fluctuation model TGF-β1 mRNA expression in (A)fluctuation down phase; (B) fluctuation up phase. All data are expressed as mean± SEM, n=3 for each treatment group. Two-way ANOVA was used to analyse the data followed by Post hoc Bonferroni, means with the same letter are not significantly different ($p>0.05$), whereas those with different letters are significantly different ($p<0.05$).

4.3.6 Impact of Iodine Fluctuation on ER Stress and Autophagy Markers GRP78 and LC3B under TSH Stimulation

After constructing the fluctuation model and collecting samples, western blot analyses were conducted on all treatment groups, samples were all from the third cycle of both the fluctuation down phase (48 h of iodine deprivation) and the fluctuation up phase (48 h of iodine incubation following 48 h of iodine deprivation). The ER protein folding mechanism, which drives misfolded proteins toward ER-associated degradation, contains one of the best-characterised chaperones, GRP78. Moreover, it is becoming increasingly obvious that GRP78 contributes to a wide range of cell injuries. To assess the potential role of the unfolded protein response in regulating the abundance of NIS protein during iodine fluctuations, we measured GRP78 expression in cell samples from the fluctuation experiment.

Employing two-way ANOVA to analyse the GRP78 protein expression during the Fluctuation Down and Fluctuation Up phases separately revealed distinct outcomes. In the Fluctuation Down phase, the interaction between iodine level and TSH ($p = 0.51$). However, the TSH level significantly affected GRP78 expression ($p < 0.001$), suggesting TSH independently influence GRP78 expression in this phase (Figure 4.11 A).

Conversely, during the Fluctuation Up phase, the reintroduction of iodine did not produce a statistically significant interaction between TSH level and iodine level ($p = 0.705$). Additionally, no statistically significant effect on GRP78 protein expression associated with TSH level ($p = 0.14$), nor iodine level ($p = 0.148$) was observed. This indicates that neither TSH nor iodine levels significantly impact GRP78 expression during the Fluctuation Up phase (Figure 4.11 B).

In summary, TSH significantly affects GRP78 expression during the Fluctuation Down phase but not during the Fluctuation Up phase. In contrast, iodine levels do not significantly influence GRP78 expression in either phase nor does the interaction between TSH and iodine level.

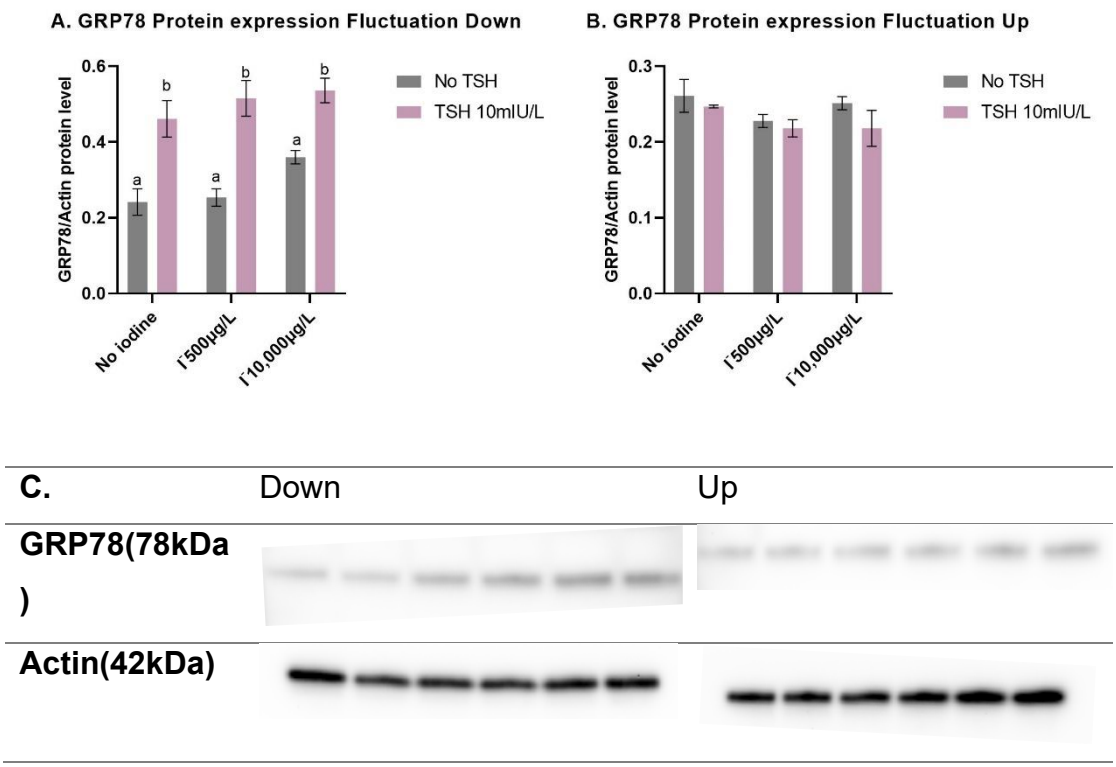


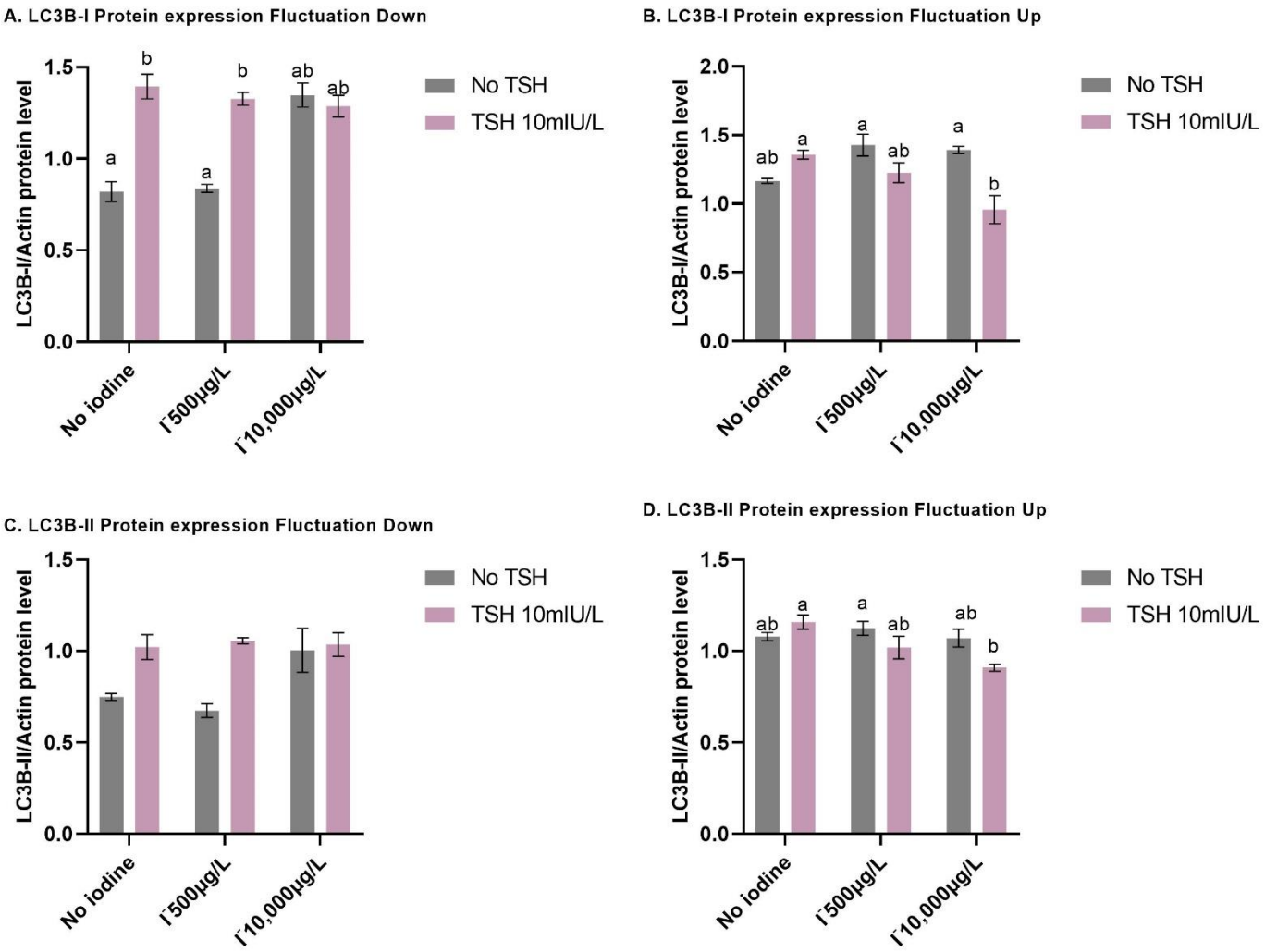
Figure 4. 11 **GRP78 protein expression in the two phases of the fluctuation cycle**

Nthy-ori 3-1 cell samples were collected during the third cycle of the fluctuation model, both after the 48 hours of iodide starvation timepoint and after the 48 hours incubation of iodide aid stage (here referring to Fluctuation down phase and up phase, respectively). Group collected include control, iodide 500μg/L only, iodide 10,000μg/L only; 10mIU/L TSH only, 10mIU/L TSH with iodide 500μg/L, and 10mIU/L TSH with iodide 10,000μg/L, in this order from left to right respectively. (A)&(B) Relative protein level of GRP78. (C) immunoblot image of GRP78 and b-actin. All data were shown as mean ± SEM (n=3). Two-way ANOVA was performed for Iodine x TSH, data followed by Post hoc Bonferroni, different letters are significantly different ($p<0.05$).

For the Protein expression of LC3B I and II, in the fluctuation down phase, as shown in figure 4.12.A and C, the interaction between TSH level and iodine level was significant ($p < 0.001$) for the expression of LC3BI protein, although for LC3BII the interaction was not significant ($p=0.053$). However, the TSH

levels significantly influenced LC3BII expression (p= 0.001), the effect of iodine level did not have a significant effect on LC3B2 (p = 0.07). In the fluctuation up phase, the interaction between iodine level and TSH were significantly affected the expression of both LC3BI (p=0.001) and LC3BII(p=0.031)

In summary, TSH markedly increased GRP78 expression during iodine scarcity but not after iodine replenishment. Conversely, iodine levels did not significantly affect GRP78 expression during any of the phases. Additionally, TSH and iodine fluctuations significantly modulated LC3B expression, reflecting changes in autophagy related to thyroid cell stress and recovery conditions.


A. LC3B-I Protein expression Fluctuation Down
LC3B-I/Actin protein level
1.5
1.0
0.5
0.0
a
b
a
b
ab
ab
No TSH
TSH 10mIU/L
No iodine
I˙500µg/L
I˙10,000µg/L
B. LC3B-I Protein expression Fluctuation Up
LC3B-I/Actin protein level
2.0
1.5
1.0
0.5
0.0
ab
a
a
ab
a
b
No TSH
TSH 10mIU/L
No iodine
I˙500µg/L
I˙10,000µg/L
C. LC3B-II Protein expression Fluctuation Down
LC3B-II/Actin protein level
1.5
1.0
0.5
0.0
No TSH
TSH 10mIU/L
No iodine
I˙500µg/L
I˙10,000µg/L
D. LC3B-II Protein expression Fluctuation Up
LC3B-II/Actin protein level
1.5
1.0
0.5
0.0
ab
a
a
ab
ab
b
No TSH
TSH 10mIU/L
No iodine
I˙500µg/L
I˙10,000µg/L

E.	Down	Up
LC3B I and LC3BII (14kDA and 16kDA)		
Actin(42kDa)		

Figure 4. 12 **LC3BI&II protein expression in the two phases of the fluctuation cycle**

Nthy-ori 3-1 cell samples were collected during the third cycle of the fluctuation model, both after the 48 hours of iodide starvation timepoint and after the 48 hours incubation of iodide aid stage (here referring to Fluctuation down phase and up phase, respectively). Group collected include control, iodide 500μg/L only, iodide 10,000μg/L only; 10mIU/L TSH only, 10mIU/L TSH with iodide 500μg/L, and 10mIU/L TSH with iodide 10,000μg/L, in this order from left to right respectively. (A) & (B) Relative protein level of LC3B-I. (C) & (D)Relative protein level of LC3B-II, (E) immunoblot image of LC3BI&II and actin. All data were shown as mean ± SEM (n=3). Two-way ANOVA was performed for Iodine x TSH, data followed by Post hoc Bonferroni, different letters are significantly different ($p<0.05$)

4.3.7 The Fluctuation of iodine does not shown interaction on affecting ROS level in the current model design.

To study whether the fluctuating iodine level affected the ROS level of the target thyrocyte, Nthy-ori 3-1 in this study, we implemented a fluctuation model by cyclically adding and removing iodine from the culture media. ROS levels were quantified 120 min after the addition of hydrogen peroxide (H_2O_2) at concentrations of 0.5mM and 2mM, following 48 h of exposure in both the down (no iodine) and up (with iodine) phases of the third cycle of the fluctuation model. Additionally, the ROS levels generated by thyrocytes after the fluctuation model without extracellular stress were quantified.

In the absence of H_2O_2 induction, as shown in figure 4.13.A and B, no significant interaction between TSH and iodine levels was observed in either phase of fluctuation ($p=0.17$ in the down phase, $p=0.815$ in the up phase). However, in the fluctuation down phase, significant differences in ROS levels were observed across the different iodine conditions ($p=0.025$), which disappeared in the up phase ($p=0.364$). Both iodine fluctuation phases showed significant differences in ROS levels between cells treated with and without TSH ($p<0.001$, down; $p=0.011$, up).

Upon introducing 0.5mM H_2O_2, a significant interaction between TSH presence and iodine levels was noted in the down phase ($p<0.001$), which diminished in the up phase ($p=0.849$) as shown in Figure 4.13.C and D. In addition, the effect of TSH on ROS levels remained statistically significant during the fluctuation up phase ($p=0.004$).

When the H_2O_2 concentration Increased to 2mM (figure 4.13.E and F), the ROS reading maintained similar patterns of effects as observed with 0.5mM H_2O_2. There was a significant interaction between TSH presence and iodine levels in the down phase ($p=0.002$), but not in the up phase ($p=0.925$). TSH continued to significantly influence ROS levels during fluctuation up phase ($p=0.005$).

These data suggest that TSH (10mIU/L) significantly regulates ROS levels in Nthy-ori cells, irrespective of iodine conditions. The presence or absence of iodine appeared to modulate the effects of TSH, particularly under iodine-depleted conditions, indicating potential compensatory stress responses or memory effects. Conversely, in the presence of iodine, the modulatory interaction disappeared. Additionally, as shown in Figures 4.14 and 4.15, there were no visual differences between the different phases (with and without iodide treatment) of the fluctuation cycle and no visual difference between the different treatment groups.

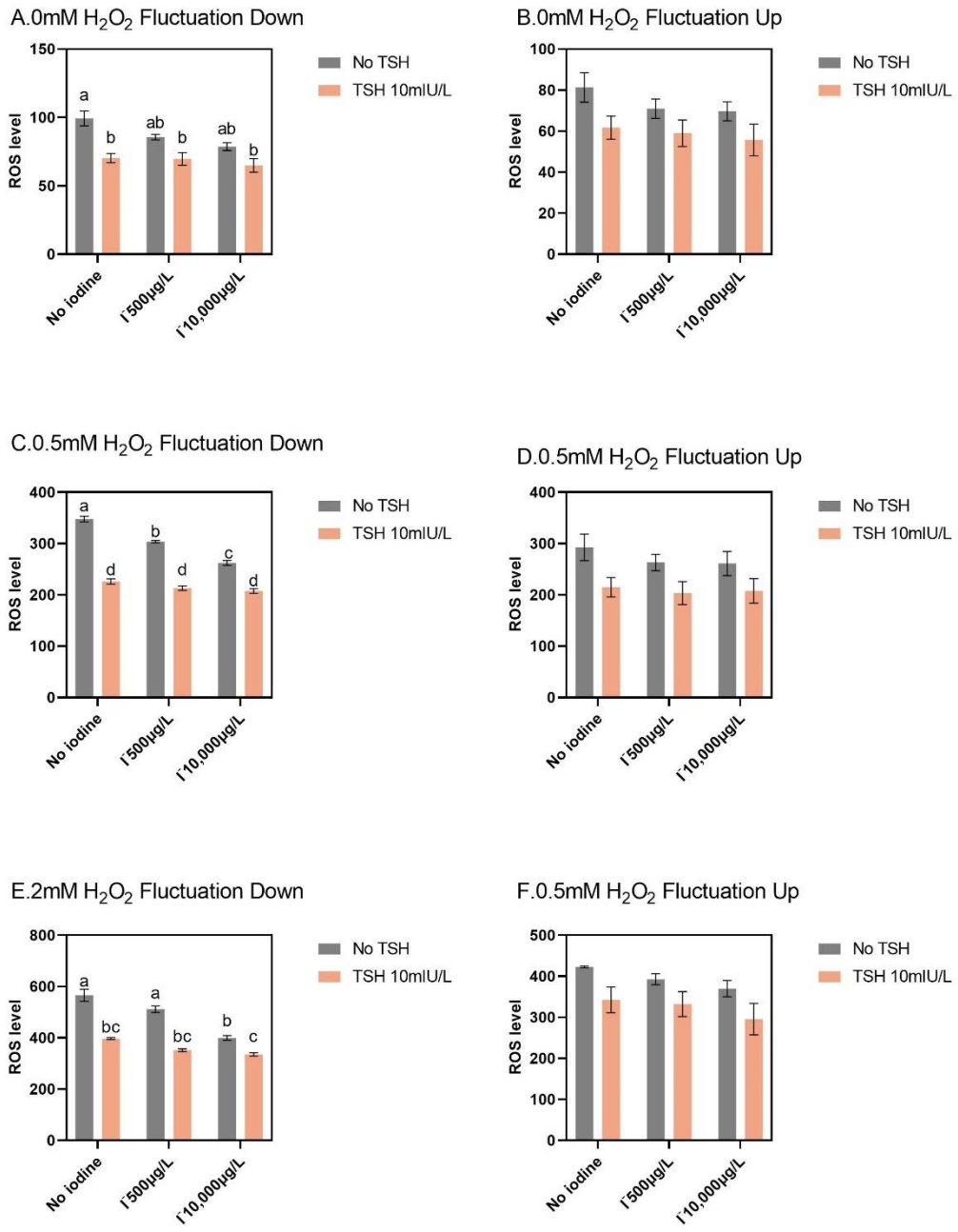


Figure 4. 13 **ROS level monitored at cycle three down and up timepoint of the Fluctuation model on thyroid cells**

The ROS level of Nthy-ori 3-1 cell in fluctuation model, (A) & (B) 0mM H_2O_2; (C) & (D) 0.5mM H_2O_2; (E) & (F) 2mM H_2O_2. All data are expressed as mean± SEM, n=3 for each treatment group. Two-way ANOVA was used to analyse the data followed by Post hoc Bonferroni, means with the same letter are not significantly different ($p>0.05$), whereas those with different letters are significantly different ($p<0.05$).

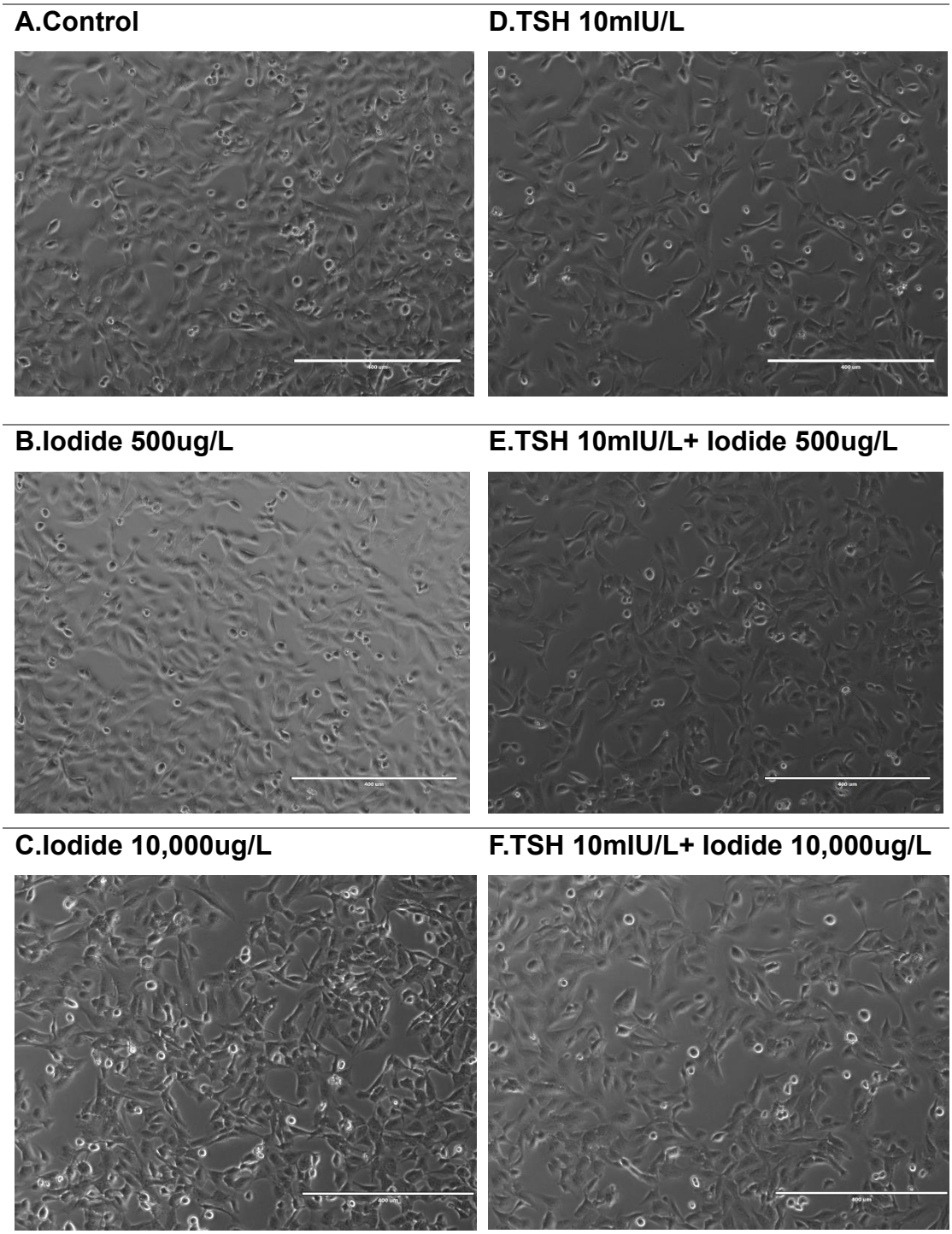


Figure 4. 14 **Effect of iodide fluctuation at the down point with Nthy-ori 3-1 cell**

Image of Nthy-ori 3-1 cell incubated with the fluctuation cycle at the stage of no iodide treatment added to the culture media for 48 hours. A) Control(No iodide; no TSH); B) Iodide 500ug/L; C) Iodide 10,000ug/L; D) TSH 10mIU/L; E) TSH 10mIU/L+ Iodide 500ug/L; F) TSH 10mIU/L+ Iodide 10,000ug/L; Image taken 10x magnification. Scale Bar =400um

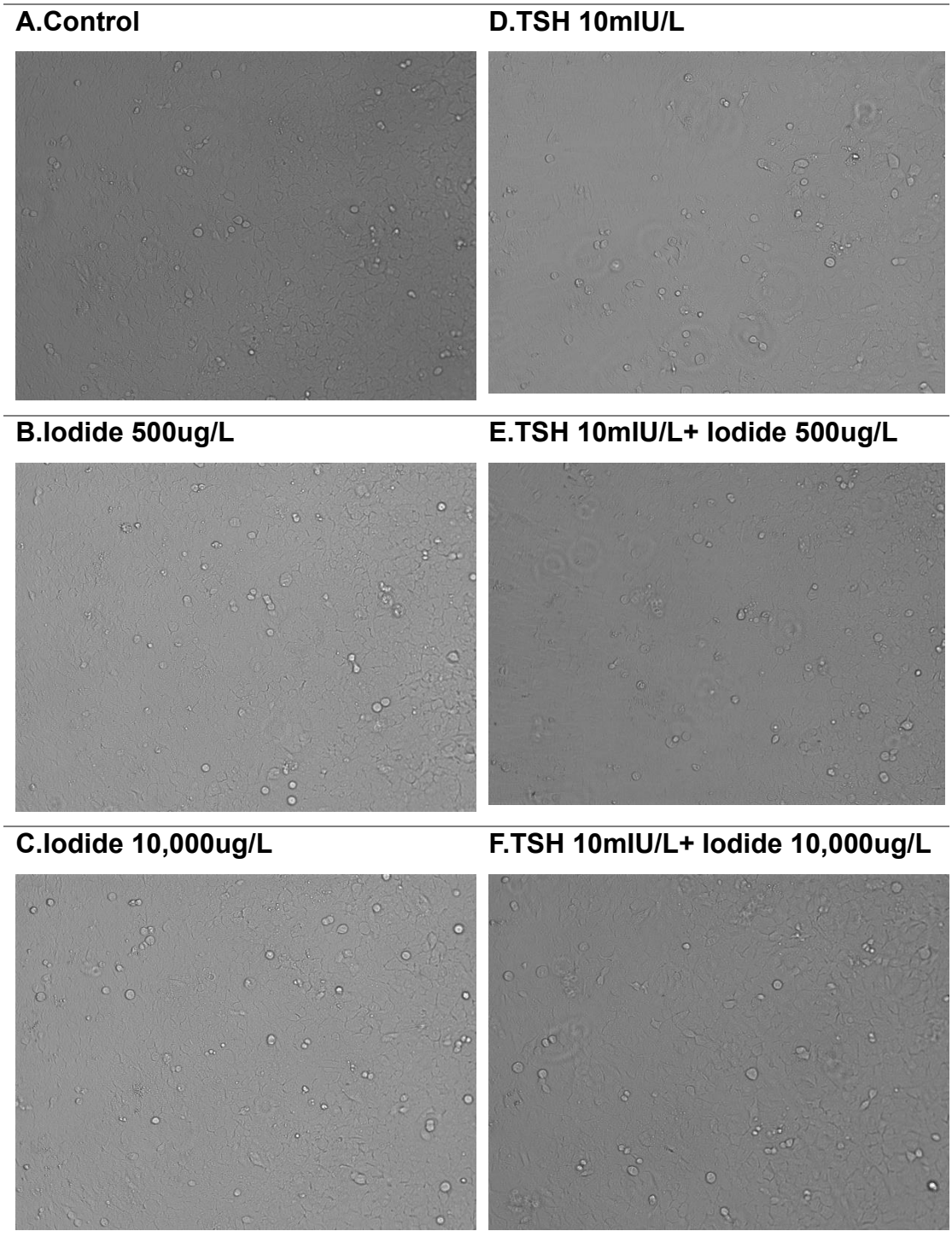


Figure 4. 15 **Effect of iodide fluctuation at the up point with Nthy-ori 3-1 cell**

Image of Nthy-ori 3-1 cell incubated with the fluctuation cycle at the stage of additional iodide treatment added to the culture media for 48 hours. A) Control (No iodide; no TSH); B) Iodide 500ug/L; C) Iodide 10,000ug/L; D) TSH 10mIU/L; E) TSH 10mIU/L+ Iodide 500ug/L; F) TSH 10mIU/L+ Iodide 10,000ug/L; Image taken 10x magnification.

4.3.8 The continuous of iodine incubation shown interaction on affecting ROS level in the thyroid cell model

For comparison with the fluctuating iodine model, another experiment of continuous iodine incubation was also conducted. As shown in figure 4.16 A, without extracellular oxidative stress induced by H2O2, the ROS level of Nthy-ori 3-1 cell did not interact with the incubation time (days) iodine level and TSH level ($p=0.838$). However, there was a two-way interaction between iodine level and TSH presence ($p=0.011$).

Additionally, the ROS level under different conditions of H2O2 exposure (0.5 or 2mM) Varys, as shown in Figure 4.16 B and C. When undertaking the 0.5mM H2O2 exposure, the ROS level of Nthy-ori 3-1 cell did not interact with the incubation time (days) iodine level and TSH level ($p=0.117$). However, all three factors were found separately (time × iodine level, $p<0.001$; time × TSH level, $p<0.001$; iodine × TSH, $p=0.001$). When H2O2 exposure increased to 2mM, a three-way interaction was found to affect ROS levels ($p<0.001$).

As a result, both iodine and TSH independently contribute to the dynamics of ROS production in thyroid cells. Furthermore, there seemed to be a peak in ROS levels around day 4, indicating a response to the treatment that increased oxidative stress in the cells.

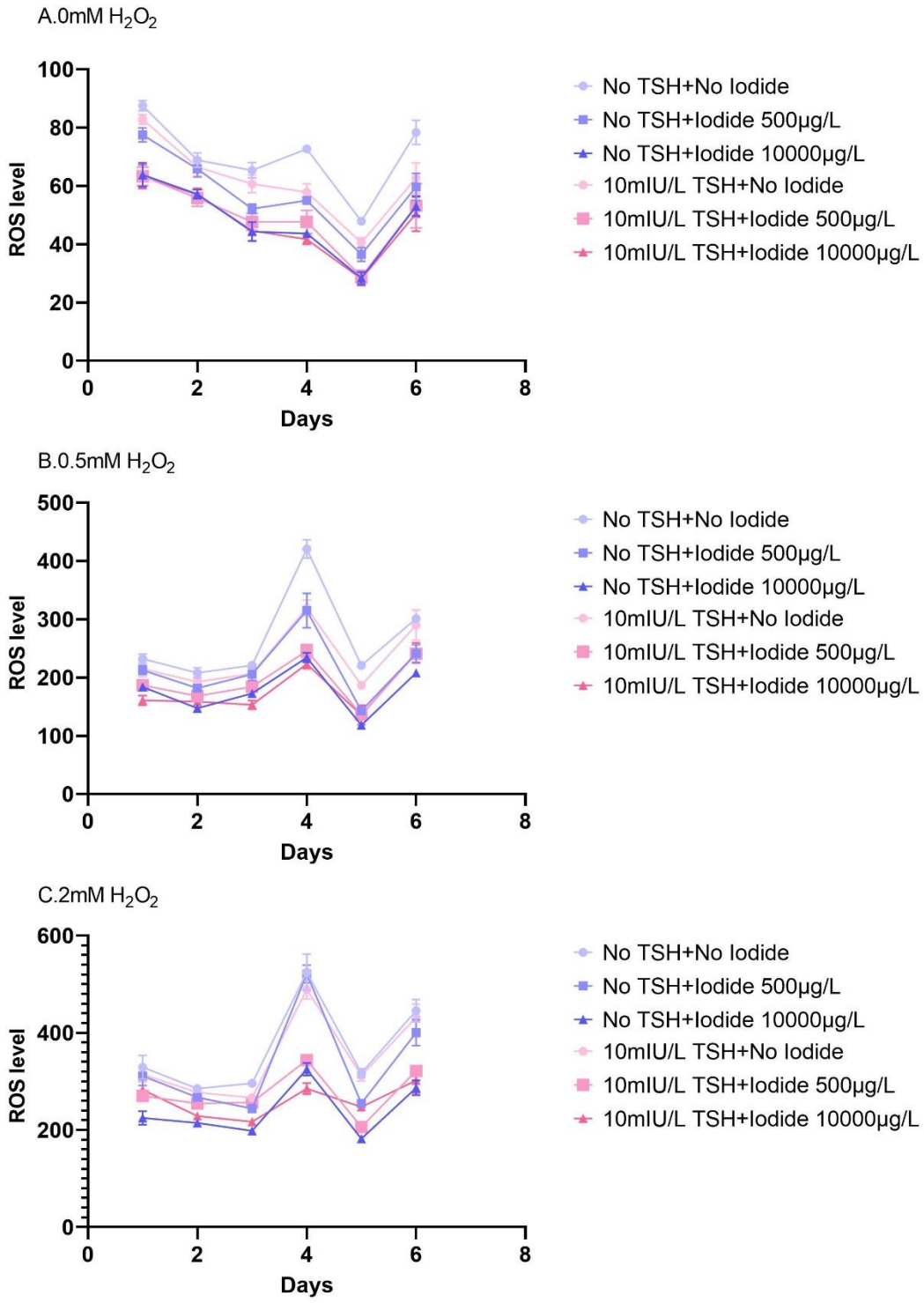


Figure 4. 16 **ROS level with continuous incubation of TSH(+/-) and iodide (+/-) on thyroid cells**

The ROS level of Nthy-ori 3-1 cell, (A) 0mM H_2O_2; (B) 0.5mM H_2O_2; (C) 2mM H_2O_2. All data are expressed as mean± SEM, n=3 for each treatment group. Three-way ANOVA was used to analyse the data followed by Post hoc Bonferroni. $P<0.05$ was considered as significant.

4.3.9 Assessment of the transwell model system to explore thyroid cell responsiveness to iodine fluctuations.

Our original intention was to employ the transwell system in order to model the thyroid cell response to iodine fluctuations, as this would permit the formation of a polarised epithelium similar to that seen in a thyroid follicle.

To determine the success of the in vitro thyroid follicle model, we assessed its ability to produce TH in response to TSH. An ELISA assay was performed to measure the level of fT4 in culture medium B after 48 h of treatment, as shown in figure 4.3A. Additionally, culture medium A from the same Transwell insert unit was also collected. However, that there was no detectable amount of fT4 in either culture media A or B.

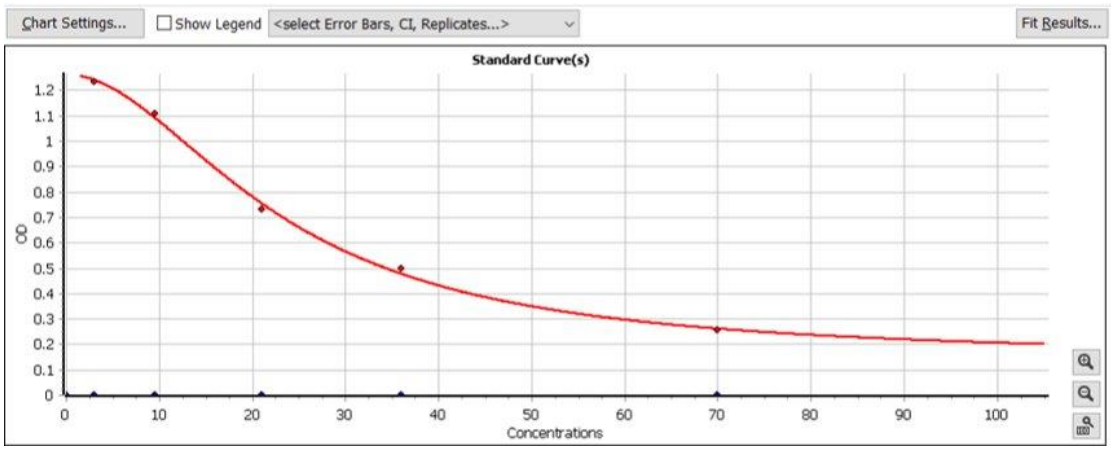


Figure 4. 17**standard curve of the ELISA assay**

Standard Curve of the ELISA assay for fT4 test for the transwell chamber.

	Blank corrected based on Raw Data (450)	fT4 Conc.
Sample 1(A)	1.842	n.a.
Sample 2(B)	1.963	n.a.
Sample 3(A)	1.848	n.a.
Sample 4(B)	1.822	n.a.
Sample 5(A)	1.955	n.a.
Sample 6(A)	1.849	n.a.
Sample 7(B)	1.816	n.a.
Sample 8(B)	1.878	n.a.
Sample 9(A)	1.791	n.a.
Sample 10(B)	1.841	n.a.

Table 4. 2 **Result OD value reading and fT4 concentrations in transwell model using ELISA**

4.4 Chapter Discussion

Since this is the first time that we are handling the normal human thyroid follicular cell line, Nthy-ori 3-1, the iodine tolerance level of the target thyrocyte was measured. The results showed that culturing thyroid cells with 2000 mg/L iodide for 48 h did not decrease their viability. A study conducted by Zhang et al. (2023) employed higher doses of KI and found that the cell viability of the thyroid cell line Nthy-ori 3-1 in the 40mM, 60mM, and 80mM KI-treated groups was significantly lower than that of the control group ($P < 0.05$). The concentration of KI used in their study, 40mM was still higher than that used in this study (15.75mM).

In our study, we utilized a TSH concentration of 10 mIU/L for the culture of thyroid cells Nthy-ori 3-1. This decision was informed by the observation that many thyroid cell studies employ significantly higher concentrations, often as much as 1 mIU/ml (equivalent to 1 U/L) (Mulcahy et al., 1985, Grollman et al., 1986, Peter et al., 1991, Kogai et al., 1997, Eng et al., 2001, Vlasov et al., 2016, Huang et al., 2011, Kurashige et al., 2020b). We opted for a

concentration closer to the physiological range observed in healthy adults, typically between 0.5 to 5 mIU/L according to UCLA Health. Considering the potential differences between in vivo and in vitro systems, such as the reduced sensitivity of immortalized cell lines like Nthy-ori 3-1, we reasoned that a modest elevation above the upper limit of the normal in vivo range might be necessary to adequately stimulate cellular activity. This approach seeks to bridge the gap between physiological relevance and the practical realities of cell-based experimentation.

Transwell model

Unfortunately, our attempt to establish a transwell model using Nthy-ori 3-1 cells did not meet with success. The cells were seeded on the transwell insert membranes and cultured for one week, after which the media in both the upper and lower chambers were replaced with treatment media devoid of FBS and standard culture media, respectively (Figure 4.3). However, 48 hours following this change, sample analysis from both chambers revealed undetectable levels of free T4, leading us to proceed with the remainder of the study without employing the transwell inserts. This outcome highlights the difficulties associated with constructing a functional transwell model, as also reflected in the work of Huang et al. (2011), who encountered challenges in forming follicular units and storing iodinated organic compounds within the follicular lumen of their transwell model (upper chamber of transwell insert) using pig primary thyroid cells. It is conceivable that our model experienced similar issues as reported by Huang et al., or that the transwell membrane had not achieved full confluence at the time of treatment administration.

Aside from stated above, there are several potential reasons for our failure to achieve a successful transwell model or to accurately determine the functionality of our model. One possible explanation is that our standard curve may have been well constructed, but it did not include culture media, which could have affected the accuracy of our results. Additionally, the design of the transwell insert may have presented challenges in terms of observation under the microscope, making it difficult to obtain direct evidence of the number of seeded cells within the insert and whether they had achieved full coverage.

Furthermore, the presence of phenol red in the culture media may have had an impact on the results, as the Elisa assay relies on fluorescence signal detection. In this study, we refrained from using the transwell model despite its potential as a valuable model system. Therefore, it is essential to address these factors in future studies to ensure the reliability of our transwell model.

Expression changes with the fluctuation model

In our study, we observed that TSHR mRNA expression was significantly influenced by both TSH and iodide, which is consistent with findings from previous studies(Huang et al., 2016, Kleinau et al., 2010, Zoenen et al., 2012, Kleinau et al., 2017). TSHR regulation by TSH is well documented, but our findings highlight that iodide can also modulate the expression of TSHR. This is similar to the research conducted by D'Agostino et al. (2014), who suggested a feedback mechanism that maintains thyroid hormone synthesis equilibrium. This further demonstrates the complexity of thyroid regulation beyond the traditional TSH-TSHR axis.

Furthermore, our results indicated that the mRNA expression of PAX8, a critical transcription factor in thyroid development and function, appears to be influenced solely by TSH in the absence of iodide. This suggests that PAX8 expression is independent of iodide levels(Jing and Zhang, 2022). The selective response of PAX8 mRNA to TSH, regardless of iodine presence, delineates the distinct pathways through which thyroid function and development are regulated(Mansouri et al., 1998, Huang et al., 2016). However, since the expression of the TSH-TSHR axis can be modulated by iodine, the interaction we discovered during the fluctuation phase, when iodine was reintroduced into the in vitro model, was reasonable.

In our study, TPO expression was significantly influenced by both TSH and iodide levels ($P = 0.052$) during the iodine deprivation phase. This result indicates a near-significant interaction and suggests a potentially intricate relationship between iodine and TSH levels, particularly during this phase of our fluctuation model. This observation is critical, given that TPO is essential for iodine oxidation and organification within the thyroid gland. Previous

studies, such as those conducted by D'Agostino et al. (2014), Xu et al. (2011) and Montesinos et al. (2016)have highlighted the regulatory roles of TSH and iodine on thyroid-specific genes, including TPO. These studies support the notion of a feedback mechanism that balances thyroid hormone synthesis under various iodine conditions. Our findings align with these observations, particularly upon reintroduction of iodine into the culture environment, where the combined presence of TSH and iodine significantly influenced TPO mRNA expression. Notably, this effect was more pronounced at excessive iodine levels (Figure 4. 9 D), suggesting a feedback mechanism where elevated iodine levels, coupled with TSH stimulation, may enhance TPO expression to regulate the intracellular iodine concentration. However, this method assumes that the levels do not exceed a certain threshold. This is in line with the findings of Eng et al. (1999) and Xu et al. (2011), which indicated that excessive iodine could downregulate TPO expression. Our results potentially extend our understanding of the threshold for excessive iodine that leads to TPO downregulation.

In addition, during the fluctuation phase, our results underscore the critical role of iodine in regulating PDS expression, a mechanism that suggests a compensatory response by thyrocytes to changes in iodine availability. The increased expression of PDS in response to iodine withdrawal indicates an adaptive mechanism aimed at enhancing iodide transport and compensating for the iodine-deficient environment. Once iodine was reintroduced, the combined presence of TSH and iodine significantly affected PDS expression, as supported by studies highlighting the differential regulation of thyroid-specific genes in response to iodine and TSH (Suzuki and Kohn, 2006, Kopp et al., 2008, Pesce et al., 2012).

Similar to PDS, the expression of DUOX2 was significantly impacted by iodide levels during the fluctuation down phase (Figure 4.9 E), the similar expression trend might suggest a compensatory response mechanism. Specifically, after being previously stimulated with excessive iodine, the thyrocytes maintained

heightened DUOX2 mRNA expression levels during subsequent iodine deficiency phases compared to cells incubated with lower iodine levels initially. This suggests that prior iodine exposure primes thyrocytes for a more active response, highlighting the persistent influence of iodine history on cellular function. Upon reintroducing iodine into the culture environment, we observed a marked interaction between TSH and iodine, which significantly influenced DUOX2 expression. This finding emphasizes a critical role of DUOX2 in responding to shifts in iodine availability, a process modulated by TSH signalling to facilitate efficient iodide oxidation and hormone synthesis (Song et al., 2010, De Deken et al., 2014). This is in line with previous studies illustrating the crucial role of DUOX2 in thyroid hormone biosynthesis and its regulation by TSH and iodine levels(Kambe and Seo, 1997, Pellizzari et al., 2000).

Expression changes with NIS

The study done by Kurashige et al. (2020b) indicates that human thyroid cell Nthy-ori 3-1 does not contain the ability to trap iodide, as their result showed that the mRNA expression of NIS was low compared to PCCL3. However, our result was different. After 48 hours of the iodide down treatment, the iodide starvation period of the fluctuation model, the protein expression of NIS was significantly higher compared to the control group but not the mRNA expression; this might indicate that during the iodide starvation, the expression of NIS was increased through the translation process.

However, our result indicates that TSH influences NIS mRNA levels (Figure 4.8.a), its effect on protein levels is less direct. The differences between the mRNA and protein expression can possibly be explained by many other factors aside from TSH. Additionally, the changes observed in the fluctuation down phase NIS protein expression might also be explained by translation delay, post transcriptional modifications or experimental faults.

The animal study conducted by Serrano-Nascimento et al. (2010) suggests that the abundance of NIS mRNA was significantly decreased following treatment with excess NaI after 30 minutes, leading to speculation that

transcript stability might be reduced. This is supported by findings that variations in the mRNA poly(A) tail length can influence its stability and translate efficiency (Munroe and Jacobson, 1990, Sachs, 1993), and their results demonstrated a reduction in the NIS transcript poly(A) tail length in the thyroid of rats treated with excess iodide. In a subsequent study utilizing the rat thyroid cell line PCCL3, also treated with excess NaI (10^-3 M) for 30 minutes, Serrano-Nascimento et al. (2012) observed an acute decrease in both NIS mRNA content and half-life following NaI treatment. Translation delay refers to a lag between the transcription of mRNA and the production of its corresponding protein. In a study conducted by Cheng et al. (2016), that cultured HeLa cell in DTT for 30 hours resulting an increase in the level of GRP78 showed an ongoing ER stress response that delayed the return to normal translation. Which the increase of GRP78 is also present in the fluctuation down phase of our study (Figure4.11).

The fluctuation with stress indicators

The buildup of unfolded or misfolded proteins in the ER is strongly correlated with the release of GRP78. The cellular reaction to ER stress is to start the unfolded protein response (UPR) to restore the ER's normal function ER and eliminate misfolded proteins, which is essential for reestablishing cellular homeostasis. The accumulation of misfolded proteins affects ER homeostasis, which leads to ER stress (Cook et al., 2016, Bravo et al., 2013). Studies has been able to connect the relationship between thyroid dysfunction and ER stress. Similar to our result during the fluctuation down phase, Zhou's study (2016) indicates that with an animal subclinical hypothyroidism model, they were able to show that TSH could be inducing hepatic ER stress. In a study done using the cellular model FRTL-5 suggested that TSH regulates the expression of GRP78 through the cAMP pathway (Endo et al., 1991). Furthermore, we observed that short-term ER stress was alleviated by the reintroduction of iodine into the environment, as shown in Figure 4.11 B. This suggests that iodine supplementation can mitigate ER stress in the short-term, potentially contributing to the maintenance of thyroid health under fluctuating iodine conditions. However, it is crucial to consider the implications of prolonged ER stress in the context of our fluctuating model. Continuous or

repeated phases of ER stress, if not adequately resolved, could detrimentally affect the overall thyroid function (Wen et al., 2017). Persistent ER stress disrupts thyroid hormone synthesis, impairs cellular homeostasis, and exacerbates inflammatory responses, leading to a deteriorated thyroid health scenario (Malhotra and Kaufman, 2007, Hetz and Papa, 2018). Therefore, although our findings indicate a temporary remedial effect of iodine re-supplementation, they also underscore the potential risks associated with sustained ER stress within the thyroid gland.

We also observed an increase in LC3B protein levels during the iodide starvation phase of the fluctuation model (Figure 4.12 A & C), particularly in cells continuously treated with TSH, as well as those exposed to high concentrations of iodine alone. This increase in LC3B, a protein associated with autophagosome formation, suggests that autophagy is induced by iodine deprivation. The elevation of LC3B in response to TSH could indicate that TSH triggers cellular mechanisms aimed at maintaining cellular homeostasis under stress conditions, such as iodide deficiency. Conversely, the reintroduction of iodide into the culture media led to a dose-dependent decrease in LC3B protein expression, consistent with the notion that adequate iodine levels may mitigate the autophagic response triggered by iodine scarcity or TSH treatment. However, cells treated with only iodide in the culture media still showed increased levels of LC3B expression, especially LC3B I and II in the 10, 000 µg/L iodide group.

This dichotomy in autophagy regulation is reflected in the literature, TSH was able to increase the autophagy, at same time thyroxine was able to suppress the autophagy in rat thyroid cell line PCCL3 (Kurashige et al., 2020a). On the other hand, one study done by Xin and colleagues (2017) suggest that in TSH-stimulated primary mice chondrocytes, the cell proliferation rate was reduced, and the apoptosis level was increased. However, the expression of autophagic marker LC3II was reduced as well as Beclin-1. It is possible that the reason of thyroxine in Kurashige's study (2020) was able to suppress the autophagy was due to the iodine component within.

In summary, TSH was found to upregulate both the ER stress marker GRP78 and autophagic marker LC3B in our study, indicating a response to cellular stress within the thyroid environment. Intriguingly, the presence of iodide appeared to mitigate these responses, as evidenced by the reduced expression levels of both markers upon reintroduction of iodine (Figure 4.11 and 4.12). This observation suggests that continuous iodine administration plays a critical role in cellular homeostasis, particularly under stressful conditions. Specifically, removal of iodine from the culture environment seems to trigger an increase in both ER stress and autophagy markers, likely as a compensatory mechanism to counteract the absence of iodine. Conversely, when iodine was reintroduced, there was a notable decrease in both ER stress and autophagy levels, suggesting that the reintroduction of iodine helps restore cellular equilibrium. This interplay between iodine availability, ER stress, and autophagy underscores the adaptive mechanisms of thyroid cells in maintaining homeostasis under fluctuating iodine conditions. The ER stress and autophagy responses can thus be viewed as indicators of the thyroid cell's efforts to cope with iodine scarcity and re-establish a balanced internal environment upon the return of iodine. These findings highlight the complex regulatory roles of TSH and iodine in thyroid cell physiology and stress responses, further emphasising the importance of maintaining appropriate iodine levels for optimal thyroid function.

These findings indicate the critical role of iodine in regulating genes associated with thyroid cell function and emphasise the intricate interaction between TSH and iodine concentrations in modulating the expression of important genes, such as NIS, under fluctuating iodine conditions. The observed responses to different iodine levels illuminate the complex mechanisms by which thyroid cells adapt to environmental changes, underscoring the significance of iodine in thyroid health and disease. Moreover, our results validate the utility of our fluctuation model, demonstrating its effectiveness in simulating dynamic changes in iodine availability encountered by thyroid cells in vitro.

The fluctuation model with the ROS level changes.

The ROS level of the fluctuation model, as well as its ability to moderate cellular oxidative stress induced by H_2O_2, were also analysed. Remarkably, we consistently observed a decrease in ROS levels with increasing iodine content within the culture media, regardless of the iodine fluctuation phase, as shown in Figure 4.13. Intriguingly, this reduction in ROS levels persisted even during the iodine fluctuation down phase when the culture media were devoid of iodine. Furthermore, when 10mIU/L TSH was introduced along with iodine in the culture media, an even greater reduction in ROS levels was evident compared with cultures with iodine alone and no TSH (Figure 4.13). Additionally, in our continuous iodine incubation experiment, a significantly lower ROS level was noted in the excess iodide group, even in the absence of TSH stimulation (Figure 4.16). ROS levels in this scenario were comparable to those in cells cultured with excess iodine, indicating that the effect was independent of the presence of TSH.

These results highlight the antioxidative potential of iodide in the cellular environment, as it protects against free radicals and peroxides (Dijck-Brouwer et al., 2022, Smyth, 2003, Winkler, 2015). Notably, the research conducted by Küpper et al. (2008) provides valuable insights into the antioxidant properties of iodide. In their study, when kelp was subjected to oxidative stress, a defensive response was triggered, releasing iodide into the surroundings, which helped mitigate the stress. Interestingly, this effect was specific to the iodide form as there were no significant changes in the levels of iodate or organic iodine. This indicates that iodide, as a specific form of iodine, plays a crucial role in antioxidative defence mechanisms within biological systems (Küpper et al., 2008).

Additionally, when only TSH present, the ROS level is significantly lower than thyroid cell without any treatment (Figure 4.13 and 4.16). Although proven by previous study that shows TSH 0.3mIU/mL increase the H_2O_2 generation (ROS), and cause DNA damage(Kyrilli et al., 2019). Other researchers also

specified that thyroid cells are perpetually subjected to the effects of ROS, in response to the physiological stimulation of TSH(Kim et al., 2001).

This phenomenon suggests a complex interplay between iodine, TSH, and ROS regulation in the thyroid cells. In addition, we previously observed that the autophagy marker (LC3B) was up regulated in the presence of TSH, during the iodine starvation phase. Giving the role of autophagy is essential for cellular homeostasis (Rubinsztein et al., 2005, Levine and Klionsky, 2004), as the process of autophagy serving to eliminate misfolded or damaged proteins and oxidative stress (Rashid et al., 2015, Wible and Bratton, 2018). Hence, it is reasonable to predict the involvement of autophagy in the downregulation of ROS levels in both the fluctuation model and the continuous incubation model. Further investigation of the molecular mechanisms underlying these observations is validated to fully elucidate the intricate relationship between iodine, TSH, and ROS dynamics in thyroid cell physiology.

Chapter 5 The effect of Thyroid hormone (T3) on target cell lines

5.1 Introduction

5.1.1 Function of thyroid hormone

Thyroid hormones, including triiodothyronine (T3) and thyroxine (T4), are essential regulators of metabolism, growth, and development throughout the human lifecycle. Their known functions can be broadly categorized into two general groups: the regulation of growth and development, and the regulation of metabolism, both at the cellular and physiological levels. The effects of THs are relevant in humans from the prenatal stage to adulthood, and affects almost every organ system, contributing to the maintenance of homeostatic balance necessary for overall health and well-being (Song et al., 2011). T4 is the primary product of the thyroid gland that secretes into the circulation; it then is deiodinated to T3 in target tissues to regulate cellular metabolism, proliferation and differentiation (Brent, 2012, Mullur et al., 2014).

THs transported in blood bound mainly to proteins due to their hydrophobic nature. The primary transport proteins, as mentioned in general introduction 1.3.3, are TBG, TTR, and albumin, with only a small fraction of THs remaining free and biologically active. TBG, produced in the liver, is the main carrier, binding 75% of circulating T3 and T4 (Braverman and Cooper, 2013). Though TTR and ALB also bind THs, they have lesser roles in TH transport; TTR is more significant in the central nervous system and in carrying retinol (Hamilton and Benson, 2001), while ALB has a lower affinity for THs(Tabachnick and Giorgio, 1964). Changes in the levels of TBG directly impact TH circulation but not thyroid function itself. The binding proteins help maintain hormonal balance and protect against rapid changes in TH levels, aiding in bodily homeostasis(Braverman and Cooper, 2013).

THs reach their target cells bound to proteins and enter through carrier-mediated transport. Recent research has clarified the significant role of specific transporters, as shown in section 1.3.3 including MCT8 and 10, OATP1C1, and SLC17A4, in facilitating the cellular uptake of THs, challenging the notion of mere diffusion and highlighting the complexity of TH transport and regulation within tissues. Both the transport though circulation and carrier-mediated transport into target cell, thyroid hormone transporters play a vital role in maintaining thyroid hormone homeostasis, regulating thyroid hormone

levels in different tissues, and ensuring proper physiological function throughout the body.

Once in the cell, the main effects of T3 on target cells by influencing transcription processes. Upon entering the target cells, THs function by acting as transcription factors, as they bind to thyroid hormone receptors (TRs) to form a complex which is able to bind to the DNA directly at the sequence session called the thyroid response element (TRE) as shown in Figure 5.1. By binding to TREs, TRs regulate the expression of target genes and modulate their transcription by their transcriptional activity. TH affects a wide range of cellular pathways and functions, and ultimately influences various physiological processes. Notably, the effects of T3 on gene expression have been demonstrated through cDNA microarray analyses in mouse liver cells, revealing alterations in genes related to metabolism, insulin signalling, and cell proliferation (Feng et al., 2000).

There are four main isoforms of the TRs, TRa1, TRa2, TRb1, and TRb2, which are encoded by the TRA and TRB genes (Cheng, 2000, Weiss and Ramos, 2004). Research has indicated that both TRa and TRb regulate a large proportion of target genes that overlap (Chan and Privalsky, 2009). The activation or inhibition of TRs could depend on the TRE, or it could depend on the presence of T3(Yen et al., 2006). However, how this process is regulated remains unclear.

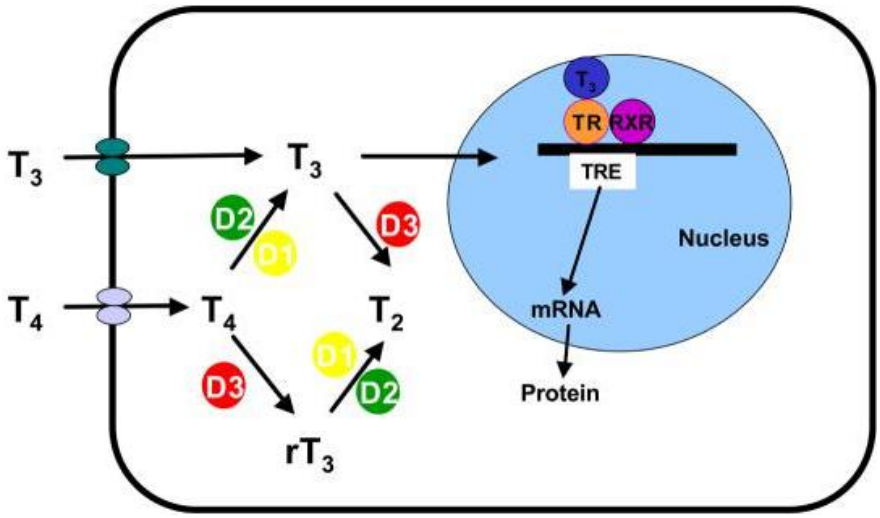


Figure 5. 1 **Thyroid hormone transport, metabolism and action in a target cell. Reproduced from Schwartz and Stevenson (2007)**

Deiodinases interact with T3 and T4 as they enter cells. The active form, T3, defuses the nucleus where it interacts with thyroid hormone receptors (TRs) to activate the synthesis of specific proteins. D2 is mostly expressed in the brain, anterior pituitary, skeletal muscle, CNS and heart, whereas D1 is primarily expressed in the liver, kidney, and thyroid. D3 is predominantly expressed in CNS, skin, and placenta. D1, D2, D3, deiodinases; RXR, retinoid X receptor; T4, T3 thyroid hormone (TH); TR, thyroid hormone receptor; TRE, thyroid hormone responsive element.

5.1.2 Thyroid hormone and oxidative stress

It is commonly known that THs act as key regulators of cellular metabolism; the metabolic effects of THs accelerates the baseline metabolism, contributing to both catabolic and anabolic responses(Schwartz and Oppenheimer, 1978). THs increase the levels of several enzymes involved in the catabolism of glucose, lipids, and proteins. In addition, the metabolic effects of THs are linked to ROS production and oxidative stress. As a result, THs are able to increase ROS production in most tissues (Messarah et al., 2011, Venditti and Meo, 2006). Although THs do not directly affect mitochondrial respiratory status, they stimulate the breakdown of ATP through various energy-consuming processes in the cell (such as thermogenesis) (Dauncey, 1990), and increase the availability of ADP. During this stage, the ROS production decreased. However, THs also increase fuel availability and

extramitochondrial ATP and NADH generation, which in turn stimulates the depletion of ADP and lowers the constituents of the mitochondrial respiratory chain(Horrum et al., 1985). The production of respiratory chain components has also been demonstrated to be stimulated by THs, enhancing the reductive state(Horrum et al., 1985, Venditti et al., 2003). It is expected that ROS production will increase under these conditions. THs also play the role of affecting the antioxidant status of the cell, from act as free radical scavengers to reducing the oxidative damage(Oziol et al., 2001). THs can affect the expression of antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). These enzymes play a crucial role in neutralising reactive oxygen species (ROS), thereby preventing oxidative damage to cellular components. On the other hand, the decrease of TH and its activity can result in reduce ROS production along with decrease the antioxidant activity from both enzymatic and nonenzymatic (da Rosa Araujo et al., 2010, Sahoo et al., 2008).

In humans, numerous chronic diseases, including cancer, type II diabetes, arteriosclerosis, chronic inflammatory processes, and cardiovascular malfunction, accelerate the pathogenesis and progression of oxidative stress.

5.1.3 Oxidative Stress Association of Hypothyroidism

Hypothyroidism is one of the most commonly clinically diagnosed thyroid disorders and is referred to as an underactive thyroid. At this stage, a dysfunctional thyroid gland decreases the production and secretion of THs, thereby increasing TSH secretion. Although hypothyroidism can be triggered by a disorder of the thyroid gland itself, iodine deficiency remains the most common cause of hypothyroidism (Braverman and Cooper, 2013). Aside from various clinical symptoms including weight gain, fatigue, poor concentration, depression, diffuse muscle pain, and constipation, no particular symptoms may be used to determine the presence of hypothyroidism (Canaris et al., 2013).

A report suggested that in the UK database, 2% of the population suffered from an underactive thyroid, with females having ten times greater likelihood than males (Werhun and Hamilton, 2013).Moreover, women of reproductive age had a 3.1% prevalence, while individuals older than 70-80 years had five times greater odds of hypothyroidism. The British Thyroid Association (BTA) states that subclinical hypothyroidism affects 5–10% of the population, with the female prevalence increasing with age(Okosieme et al., 2016, Chaker et al., 2017, Biondi et al., 2019) .In a study by Sohail Siddiqui and Shotliff (2019) on the prescription of hypothyroidism, levothyroxine was the third most prescribed drug in the UK(the top one was Atorvastatin) and the most prescribed drug in the US. The prescription number of the medicine has grown from 2.8 million in 1998 to 19 million in 2007 and increased to 29.7 million in 2015 in the UK (NHS prescription database 2016). Another study by Aoki et al. (2007) which included more than 4,000 participants reflecting the 222 million U.S. population between 1999-2002, found that approximately 3.7% of the general U.S. population was diagnosed with hypothyroidism (TSH > 4.5 mIU/L). This number increased to 5% in another report published in 2022, indicating that over 5% of the US population older than 12 years of age suffers from hypothyroidism(Patil et al., 2022).

Left-undiagnosed/untreated hypothyroidism may have a substantial impact on the economy, as it is one of the most prevalent endocrine disorders worldwide. Hypothyroidism affects individuals of all ages and can lead to a wide range of health issues. On the one hand, costs are caused by maternal and congenital hypothyroidism(Smallridge and Ladenson, 2001, Malik and Butt, 2008), as well as hypothyroid patients who also have other medical conditions, such as diabetes, as comorbid problems(Raval and Sambamoorthi, 2012). Lower quality of life is also linked to hypothyroidism, most commonly due to symptoms such as weight changes, fatigue, and depression(Guglielmi et al., 2018, Dew et al., 2017, Shin et al., 2016). In addition, hypothyroidism in older adults can present unique challenges as its symptoms can be subtle and mistaken for signs of aging or other health conditions(Bensenor et al., 2012). This can lead to under-diagnosis or

delayed treatment, potentially exacerbating health issues and reducing the quality of life of older individuals(Thiruvengadam and Luthra, 2021).

Evidence indicates that oxidative stress is associated with hypothyroidism (Resch et al., 2002), as the hypothyroidism can reduce the metabolic rate, which implies a reducing ROS production, but the available antioxidant level is reduced, making the body susceptible to oxidative damage.

5.1.4 Bridging iodine fluctuations with cellular responses to THs

In the complex interplay between iodine fluctuation, TH synthesis, and cellular responses, THs represent the highest point of a tightly controlled production line, from iodine uptake to hormone synthesis and action in target cells.

This intricate balance underlines the significance of understanding how fluctuations in iodine levels affect thyroid cells and their response to oxidative stress, as discussed in the previous chapter.

Moreover, THs play a crucial role in defending target cells against oxidative stress, highlighting their broader significance beyond metabolic regulation. Investigating this aspect not only deepens our knowledge of thyroid physiology but also emphasizes the critical impact of iodine across various bodily functions, reinforcing its importance in maintaining overall health.

Aside from research done with in vivo model, some in vitro models, such as the liver cell line HepG2 (Chen et al., 2016), kidney cell line HK2 (Poplawski and Nauman, 2008), and C2C12 (Sultan et al., 2006), have been used in studies examining the role of THs. However, when involved with in vitro studies, aside from HepG2, no sufficient studies have been done on the effect of TH in assisting other cell lines resist oxidative damage.

In the first instance, therefore, we set out to establish how these cells from target tissue responded to TH as well as their resistance against H2O2-induced oxidative damage with the assistance of THs. This is to set up an in

vitro study to determine how different cell lines from TH target tissues respond to oxidative damage with the involvement of TH.

We drew inspiration from two separate published papers to induce in vitro H_2O_2-mediated oxidative damage. A study conducted by Lamirand et al. (2008) observed rat astrocytes for up to 24 hours in the presence of 0.25mM H2O2. In another study conducted by Chen et al. (2016), HepG2 cells were treated with 2H of 0.3mM H_2O_2 before being treated with either T3 or T4 in order to investigate the dynamics of stress-induced TH in these cells.

5.1.5 Aim and hypothesis

The objective of this chapter was to explore the potential effects of T3 to cell from target tissue. Specifically, we examined whether T3 could enhance i) the proliferation rate of the designated cell lines and ii) the viability of thyroid hormone (TH) target cells when subjected to external oxidative stress.

Based on the findings of the mentioned studies, we modified and applied both of their oxidative stress models to construct our experimental model. Given the established role of TH, we hypothesised that T3 might contribute to an increase in both proliferation and survival rates of the designated cell lines.

5.2 Method

5.2.1 Cell culture

The detail of culturing HepG2, HK2, C2C12 and HEK293 cell lines was described in section 2.1.

5.2.2 Composition of the treatment media

To study the role of thyroid hormones (THs), serum containing all hormones must be restricted. Subsequently, T3 was manually added back to the media to create treatment media.

Three different base medium were used for this study. In this chapter, the control medium is referred to as High-glucose Dulbecco's Modified Eagle's medium (DMEM, D6546, containing 4500 mg/L glucose and sodium pyruvate without L-glutamine, Sigma-Aldrich) supplemented with 5 ml of L-glutamine and 5 ml of penicillin-streptomycin with 10% normal FBS.

Charcoal-stripped FBS (A33821, Gibco, Life Technologies Ltd) was used in this study to replace normal FBS to make the hormone-free medium; 10% of the charcoal-stripped FBS was added to DMEM (D6546, Sigma-Aldrich) supplemented with 5 ml of L-glutamine and 5 ml of penicillin-streptomycin to prepare HFM. As the FBS was charcoal filtered would eliminate most of the hormone contained within(Hou and Disinski, 1991). In addition to the HFM with charcoal-stripped FBS, another treatment medium was prepared, serum-free medium (SFM), with no serum to completely eliminate the effect of FBS.

20ug/ml of the Triiodothyronine (3,3′,5-Triiodo-L-thyronine sodium salt, T3, T5516, Sigma-Aldrich) was prepared using 1ml of 1N NaOH to gently dissolve and then again dissolved in 49ml of DMEM to prepare the stock solution. The T3 stock solution was then split into well-labeled 1.5ml tubes before being stored at -20°C.

To establish the appropriate concentration of T3 for our experiment, we consulted existing literature that has explored the physiological and biochemical effects of thyroid hormones in in vitro studies. The majority of cellular research articles show that the concentration of T3 used is 100nM(Poplawski and Nauman, 2008, Sinha et al., 2015, Czarnecka et al.,

2016, Tawfik et al., 2022, Liu et al., 2022), or a range of T3 between 10-9M and 10^{-5}M which includes 10^{-7}M(100nM) in the middle(Chen et al., 2016). These studies have provided valuable insights and evidence regarding the in vitro range of T3 concentrations that have shown biological effects, which guides our selection of the appropriate concentration to be used in our own research. As the result, in our experiment, 100nM T3 was added to the HFM and SFM to make T3-treated HFM and SFM media (HFM+T3; SFM+T3).

5.2.3 Using the cell counting equipment to analyze the effect of T3 treatment on cellular proliferation rate in different cells

HepG2, HK2, and C2C12 cells were seeded $1x10^{-5}$ in one well and grown in 6-well plates until they reached approximately 50% confluence, and the medium was changed every other day. After reaching 50% confluence, the cells were divided into three groups: control, HFM, and HFM+T3 groups. During the first 24 h, cells in both the HFM and HFM+T3 groups were cultured in HFM for 24 h. In the next 24 hours, which is from 24- 48 hours, cells in the HFM group stayed the same and the HFM+T3 group was changed into HFM+T3 media; the control group was cultured in control media for all time.

One 6-well plate contributed one well to each of the three groups, and all three groups had triplicate results in separate plates (n=3).
A diagraph representation of this protocol is shown in Figure 5.2.

At the endpoint of each phase, a 200µl of trypsin-EDTA solution (0.25% trypsin-EDTA, Gibco, Life Technologies Ltd) was added into each well for 5 min, and then 800µl of DMEM was added to the trypsinised well to terminate trypsin activity. The cell suspension was then carefully transferred to a well-labelled Eppendorf tube for the following cell count procedure.

A LUNA-II™ Automated Cell Counter (Logos Biosystems, Inc.) was used for cell counting. A 10µl cell suspension was used for cell counting, and the same amount of 0.4% Trypan Blue was added to a new Eppendorf tube and mixed

with the cell suspension gently. 10µl of Trypan Blue-treated cell suspension was added to the cell counting slides for auto cell counting. At the 24 and 48 hours timepoint, the cells in all three group were taken out for counting.

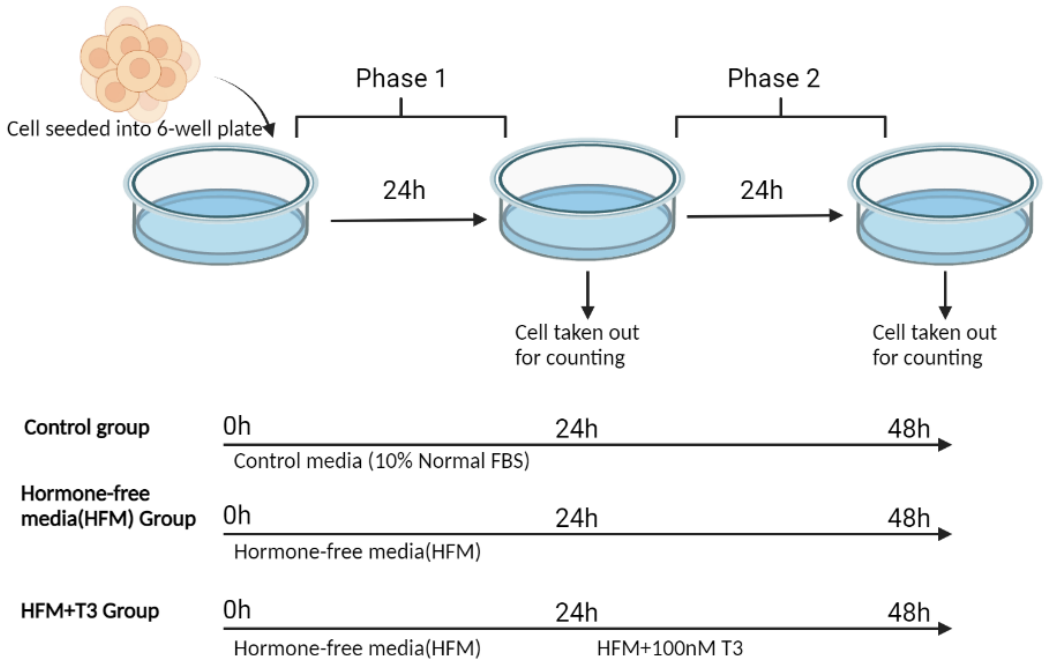


Figure 5. 2 Building the Hormone-free cellular model.

Cells were seeded onto 6-well plates around 50,000 per wells. When it reaches 50% confluence, then change the media to hormone-free media (HFM) for 24h. Afterwards, the cell was then cultured in either HFM or HFM+T3 for another 24h, a normal cell cultural media as control was also performed in all plates.

5.2.4 Investigation of the effect of T3 towards cellular stress via Cell viability assay

The concentration range used for H_2O_2 was adapted from published research papers. The majority of the H_2O_2-induced oxidative stress study employed the concentration range of H_2O_2 between 0.2mM (Chen et al., 2016)up to 0.5mM; with some studies taking the concentration to an extreme as stimulate the cell in 10mM of H_2O_2 for 2h. To apply their experience to our study, we first set the concentration range at a maximum of 2mM based on the research papers as well as the suggestion from our senior lab members. Four types of different cell lines were used in this experiment, including **HepG2, HK2, C2C12 and HEK293**.

There were two sets of protocol for the viability assay.

First protocol: the cell was seeded 10,000 cells per well and grown in the 96-well plate until it reaches 80% confluence, the culturing media was then removed. The cells were then treated with different concentrations of H_2O_2 (0, 0.2, 0.4, 0.6, 0.8, 1, 2mM), (Hydrogen peroxide solution, H-1009, Sigma-Aldrich) in two different treatment media (HFM and HFM+T3) for 24 h. Afterwards, the H_2O_2 treated treatment media was removed from all wells, and the cells were washed with 200µl PBS twice. Following washing, the cells were incubated with either HFM or HFM+T3 for another 24 h (Figure 5.3b). The same treatment for each cell line was repeated at least three times. The control was also performed on all plates(n=3).

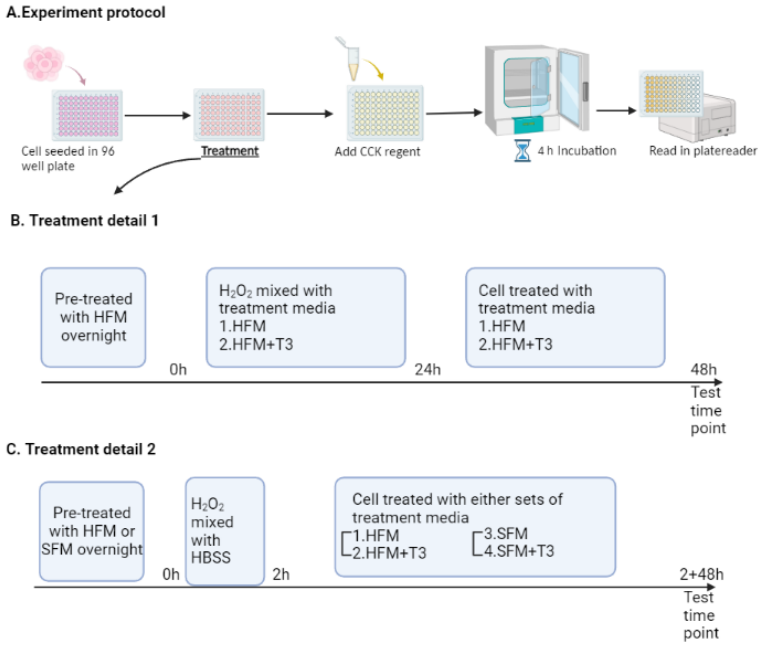


Figure 5. 3 Protocol of the oxidative stress with different treatment

A represent the CCK-8 protocol; B represent the first treatment protocol; C represent the second treatment protocol. This figure was created with BioRender.com.

Second Protocol: The cell was seeded 10,000 cells per well and grown in the 96-well plate until it reaches 80% confluence, the culturing media was then removed. Cells were treated with different concentrations of H_2O_2 (0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5mM), during this experiment protocol, aside from HK2,

H_2O_2 concentration applied to other tested cell lines was reduced to maximum of 0.5mM before being incubated in a 37°C incubator (containing 21% O_2 and 5% CO_2) for two hours. Afterwards, the cells were washed twice with PBS and maintained in serum-free media (FBS-free) or T3-treated Serum-free media. All the four cell types were incubated at 37°C with 21% O_2 and 5% CO_2 for 48 h. Each concentration of H_2O_2 was assessed in triplicate for each treatment (both serum-free or hormone-free treatment and T3 treatment) in the same plate, and the same treatment for each cell line was repeated at least three times. The control was also performed on all plates(n=3).

The associated experimental steps were same as described in detail in the section 2.2.

To assess viability, we used CCK-8. This comprises WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which can produce a yellow-colored product, formazan, which is highly water-soluble and also non-toxic. WST-8 can be reduced by dehydrogenases in cells to give formazan, which is directly soluble in the cell culture medium, and no need to add anything else. The amount of formazan dye is proportional to the number of living cells, and its sensitivity is higher than other tetrazolium salts like MTT or XTT.

5.2.5 Statistical Analysis

All statistical analyses were performed using GraphPad Prism 9.3.1. All data are expressed as mean ± SEM. Student's t-test, one-way analysis of variance (ANOVA), or two-way ANOVA was employed for the analysis. Both Student's t-test and one-way ANOVA were used for cell proliferation analysis; student's t-test and two-way ANOVA was used for cell viability analysis. If different, they will be specified in the section.
P <0.05 was considered statistically significant difference.

5.3 Result

5.3.1 Thyroid hormones (T3) are capable of inducing proliferation in HK2, HepG2 and C2C12 cells.

A simple cell counting experiment was carried out on three cell lines to test whether T3 can affect the proliferation rate of the target cell lines HepG2, HK2, and C2C12. In the cell counting assay, all cells were treated with control, HFM or HFM+T3 media, for the cell with either HFM or HFM+T3 media, the first 24h were cultured in HFM media to eliminate the effect of normal serum before the HFM+T3 group was switched to 100nM T3 treated media for another 24h.

As the first 24 h was the hormone deprivation phase, only HFM was used; hence, the results are shown separately. In our study, both HK2 and C2C12 cells showed a significant decrease in cell number after 24 h of hormone deprivation ($p<0.05$, Student's t-test). Cell numbers between the control and HFM groups of HepG2 cells did not show significant differences, with a p-value of 0.076.

There were statistically significant differences in the proliferation rate between groups, as determined by one-way ANOVA, in all three cell lines (HK2, $p=0.023$; HepG2 $P=0.002$; C2C12, $p<0.001$). The Bonferroni post hoc test revealed that after 24 h of T3 treatment, the number of HK2 cells in the HFM group was significantly lower ($p<0.05$) than that in the HFM+T3 group. No significant difference was found between the control and HFM groups($p=0.372$) or between the control and HFM+T3 groups ($p=0.117$) in the HK2 cell line.

In the HepG2 cell line, after 48h of HFM treatment, the cell number from the HFM treatment group was significantly lower than that in the control group($p<0.05$). However, no significant difference was observed between the control and HFM+T3 groups ($p=0.066$). The HFM+T3 group had a significantly higher cell number than the HFM group($p<0.05$).

The results for the C2C12 cell line indicate that after 48 h of different treatments, compared to the control group, both the HFM and HFM+T3

groups had significantly lower cell numbers (control vs. HFM, p<0.001; control vs. HFM+T3, p=0.001). In addition, the mean value of cell number after 24h of 100nM T3 treatment was more than 1.9x105 compared to the HFM group, although it did not significantly increase cell number growth compared to HFM treatment (p=0.052).

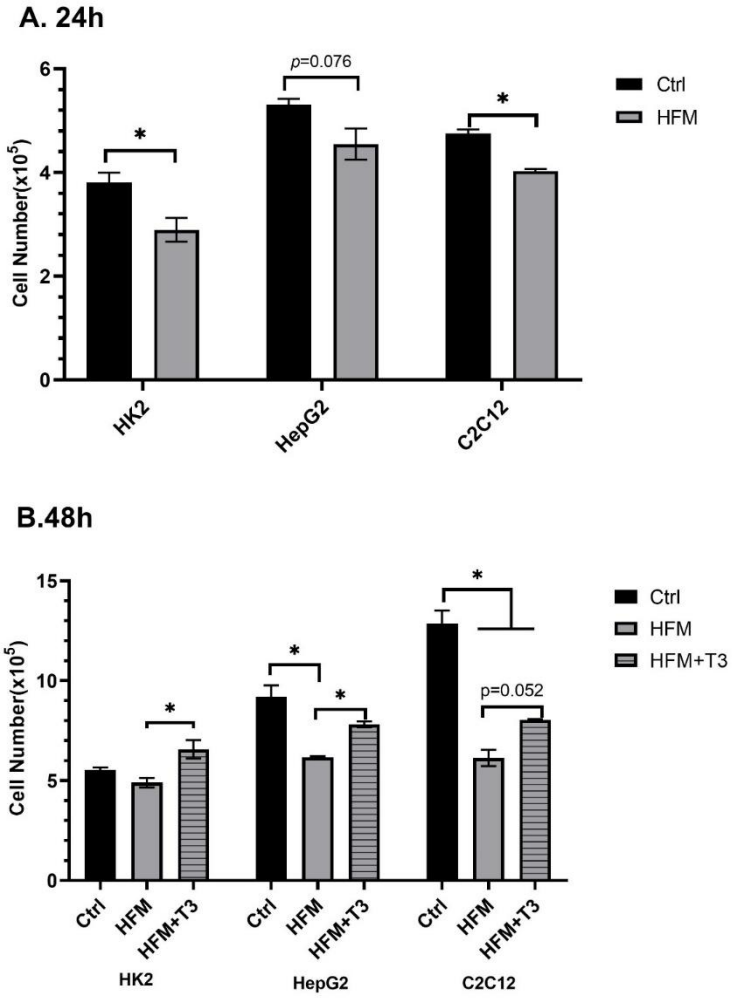


Figure 5. 4 **Effect of 24h T3 treatment on different cell lines**

Changes of cellular proliferation rate can be found when cultured cell with HFM for 24h(A); and growth rate changed again when hormone-starved cell incubated in HFM+T3 for another 24h (B). *p<0.05 was considered as statistically significant (Student T-test for 24 h; One-way ANOVA Bonferroni for 48h result). All data are expressed as the mean± SEM, n=6 for each treatment group. (ANOVA HK2 p=0.023; HepG2 p=0.002; C2C12 p<0.0001)

5.3.2 No protective effect of T3 treatment was observed with tested cell lines after 24h of T3 treatment.

Two-way ANOVA was conducted to investigate the impact of H_2O_2 concentration, ranging from 0 to 2mM, and the involvement of T3 on the viability of T3 target cell lines. In this study, three cell lines, HepG2, HK2, and C2C12, were used. All cell lines underwent a 24-hour T3 incubation phase (T3 or no T3 groups) before being cultured in a range of H_2O_2 concentrations for 2 h.

Two-way ANOVA demonstrated that in the HepG2 cell line, there was no statistically significant interaction between T3 treatment and H_2O_2 concentration ($P = 0.055$). However, as shown in Figure 5.5 A, H_2O_2 levels had a statistically significant impact on the viability of the T3 target cell lines ($p<0.001$). According to the post hoc Bonferroni analysis, the cell viability of HepG2 cells was found to be significantly decreased after exposure to 0.2mM of H_2O_2 stress for 2 h, while no statistically significant change was observed in cell viability after exposure to 0.6mM or higher concentration of H_2O_2. Additionally, T3 treatment did not have a significant effect on cell viability ($p=0.414$).

Similarly, the results of the two-way ANOVA for the HK2 cell line indicated that there was no statistically significant interaction effect between the T3 treatment and H_2O_2 concentration ($p=0.871$). Although H_2O_2 stress had a statistically significant effect on the viability of HK2 cells ($p<0.001$), and the post hoc Bonferroni test indicated that the concentration of H_2O_2 that significantly decreased HK2 cell viability was located between 0.5mM to 1mM (Figure 5.5 B). T3 treatment had no statistically significant impact on cell viability ($p=0.209$).

Similarly, the results of the two-way ANOVA for the C2C12 cell line indicated that was no statistically significant interaction between T3 treatment and H_2O_2 concentration in C2C12 cells with the treatment used ($P = 0.632$). The H_2O_2 level had a statistically significant effect on the viability of C2C12 cells ($p<0.001$). Based on the results of the post hoc Bonferroni analysis, the

viability of C2C12 cells was significantly reduced after exposure to 0.25mM of H_2O_2 stress for 2 h (Figure 5.5 C), whereas no statistically significant change was detected in cell viability following exposure to 1mM or higher concentrations of H_2O_2. The cell viability was not significantly affected by T3 treatment (P =0.059).

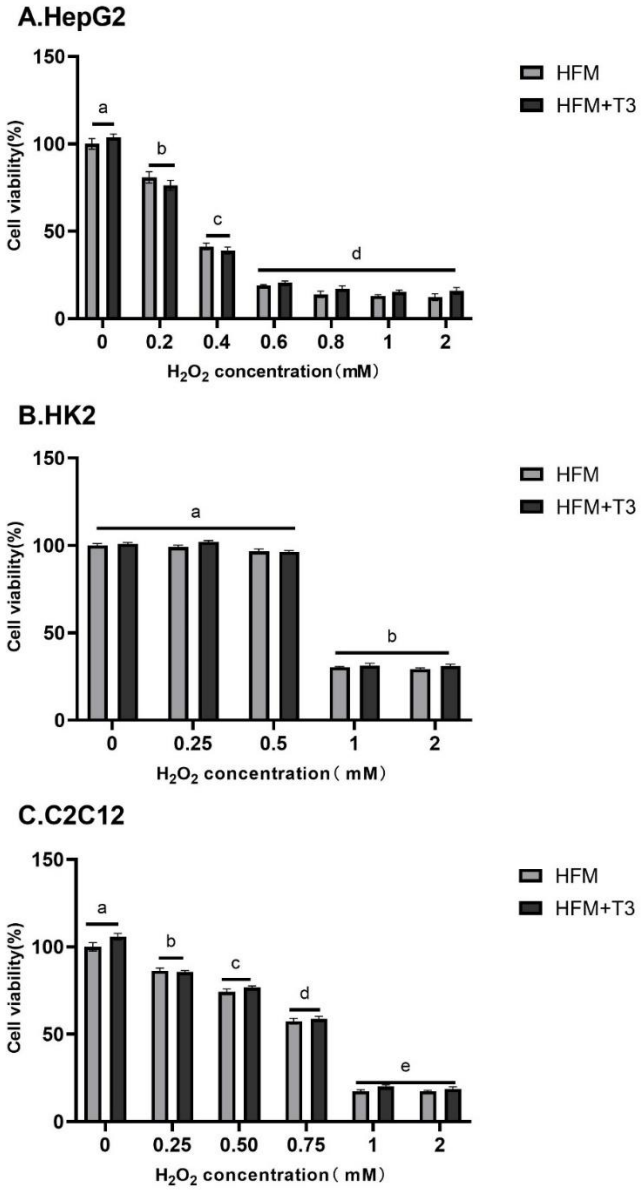


Figure 5. 5 **Effect of H2O2 induced cellular stress**

All data are expressed as the mean± SEM, n=6 for each treatment group.

Two-way ANOVA was used to analyse the data followed by Bonferroni, Means with the same letter are not significantly different ($p>0.05$), whereas those with different letters are significantly different ($p<0.05$).

5.3.3 Compare the difference of HFM and SFM media

To eliminate the effect of serum as much as possible to investigate the protective effect of T3 on target cells, a simple cell viability assessment was conducted. This was done to assess survivability in both experimental culture environments (culture media) in the targeted cell lines, HK2, HepG2, and C2C12.

All three cell lines tested were incubated with either hormone-free media (HFM, using charcoal-stripped FBS) or serum-free media (SFM, using no FBS) continuously for 48h; CCK-8 kit was used to assess the cell viability. For comparison, the cell viability with HFM was set to 100%, and the cells cultured with SFM were then calculated accordingly.

As shown in Figure 5.6, the percentage of cell viability of HK2 cultured with SFM only showed a slight drop change of 97.49% compared to cells cultured in HFM. The viability of HepG2 cells decreased by approximately 10% when they were cultured in SFM for 48h. In contrast, the viability of C2C12 cells cultured in SFM dropped sharply; only 19.46% of cells were viable compared to 100% in HFM. This result showed that the SFM medium did not support the maintenance of the viability of the C2C12 cell line, unlike HK2 or HepG2 cells. Hence, for the following part of this chapter, we will use the SFM for both HK2 and HepG2 as base culture media, as C2C12 remains in the HFM medium.

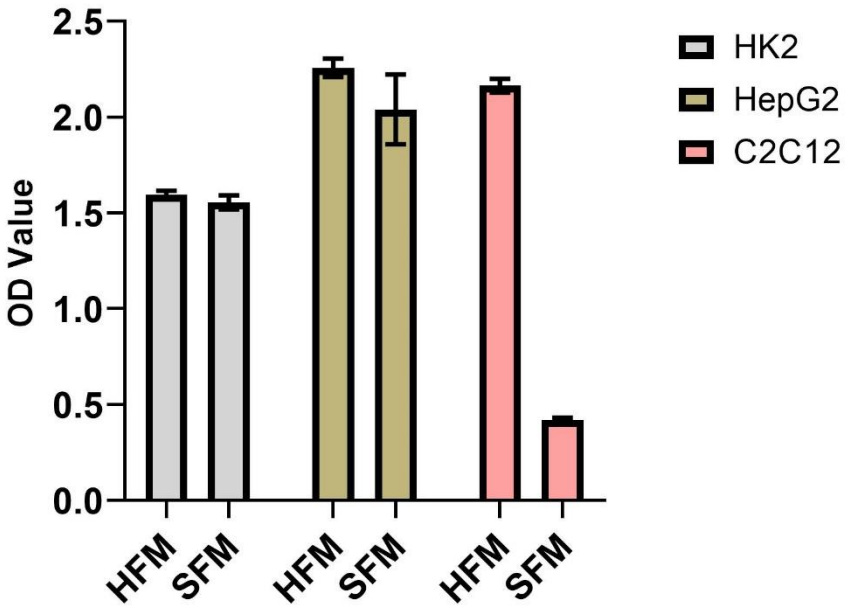


Figure 5. 6 **Cell viability comparison with target cell line.**

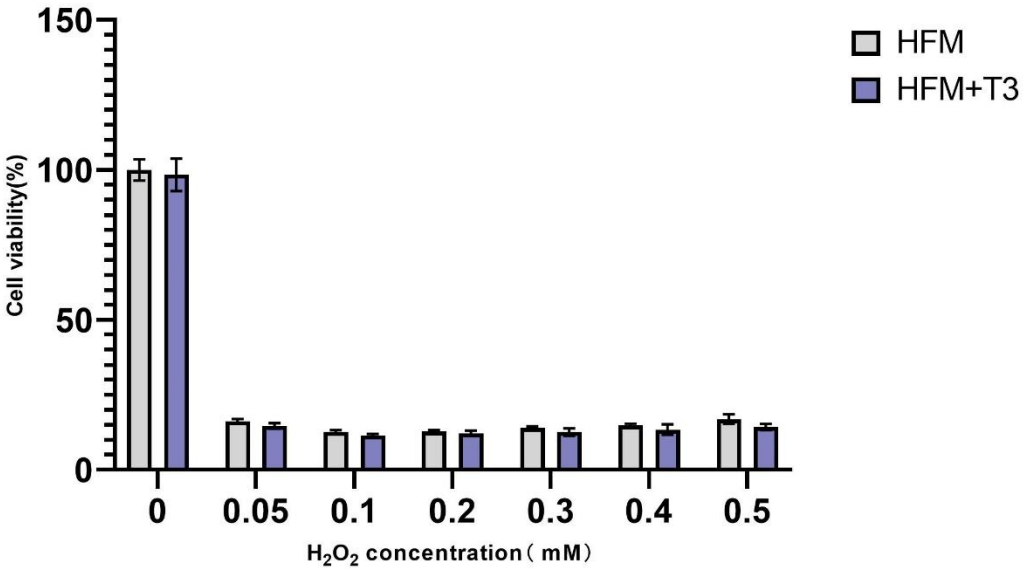
All data are expressed as the mean± SEM, n=3 for each treatment group.

5.3.4 TH have protection effect against H_2O_2 on target cell line HepG2 but no other tested cell lines

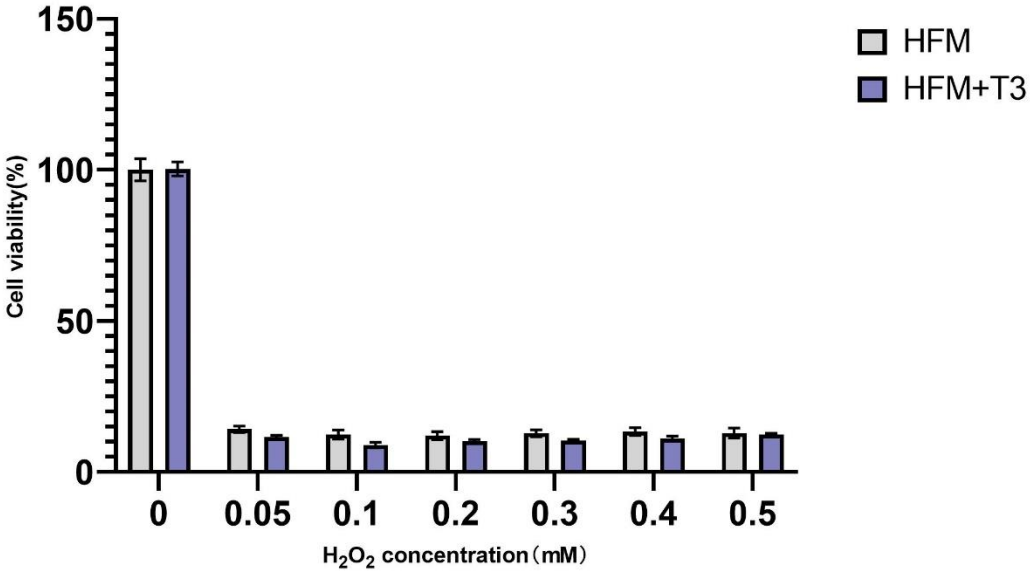
To investigate whether culture in a hormone-free medium or serum-free medium and adding 100nM T3 back to the treatment medium could assist the cell's ability to endure different levels of hydrogen peroxide (H_2O_2)-induced oxidative stress, the CCK-8 assay was carried out on all three cell lines with HK2, HEPG2, and C2C12. All cell lines were treated with an increased concentration of H_2O_2 (from 0-0.5mM with HepG2 and C2C12; 0-2mM with HK2 cell line) for two hours before being cultured in either SFM or T3-treated SFM for another 48h (HFM for C2C12 as suggested in the previous section). Viability assay was conducted right after the 48h incubation.

The analysis done with Two-way ANOVA showed that no interaction between T3 treatment and H_2O_2 was observed ($p=0.183$ 24h H_2O_2 x T3 treatment; $p=0.788$ 48h H_2O_2 x T3 treatment), the cell viability of C2C12 decreased significantly with the involvement of H_2O_2 ($p<0.001$) after both 24h and 48h of the recovery phase. Post hoc Bonferroni test indicated that 0.05mM H_2O_2 significantly decreased C2C12 cell viability, and T3 treatment did not make any difference ($p=0.133$ T3 treatment at 24h; $p=0.906$ at 48h). C2C12 cells were observed and imaged under a microscope, as shown in Figure 5.7.C. Only cells that were not challenged with H_2O_2 showed visible live cells; most of the visual sites of other groups showed no signs of viable cells, regardless of the H_2O_2 concentration or either HFM or T3-treated medium incubation time.

a.C2C12 24h



b.C2C12 48h



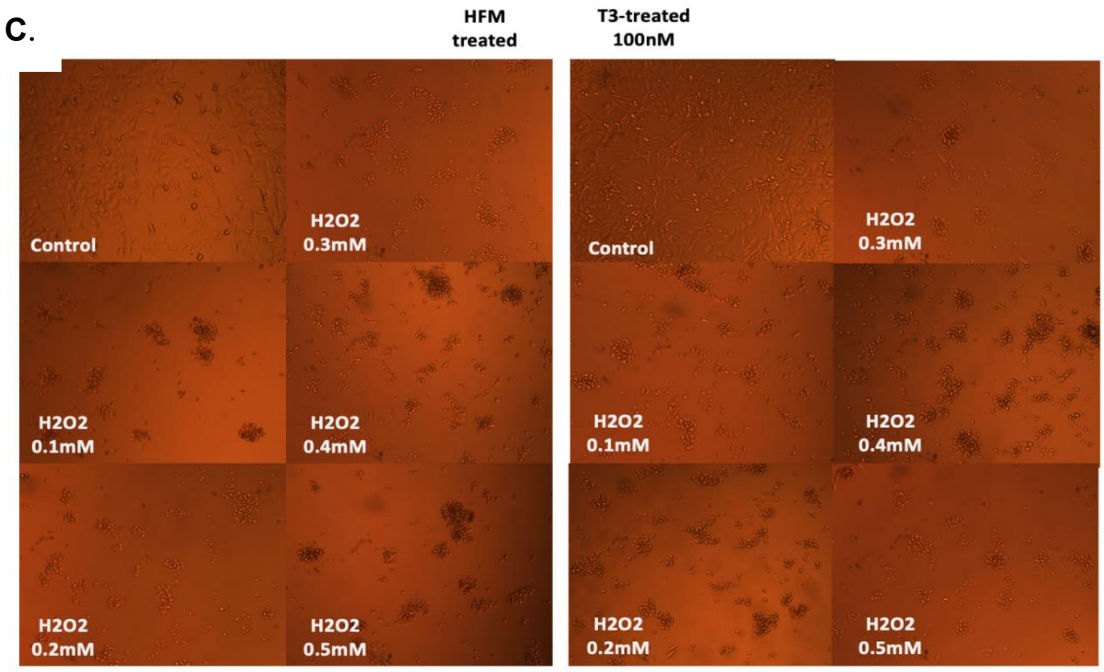
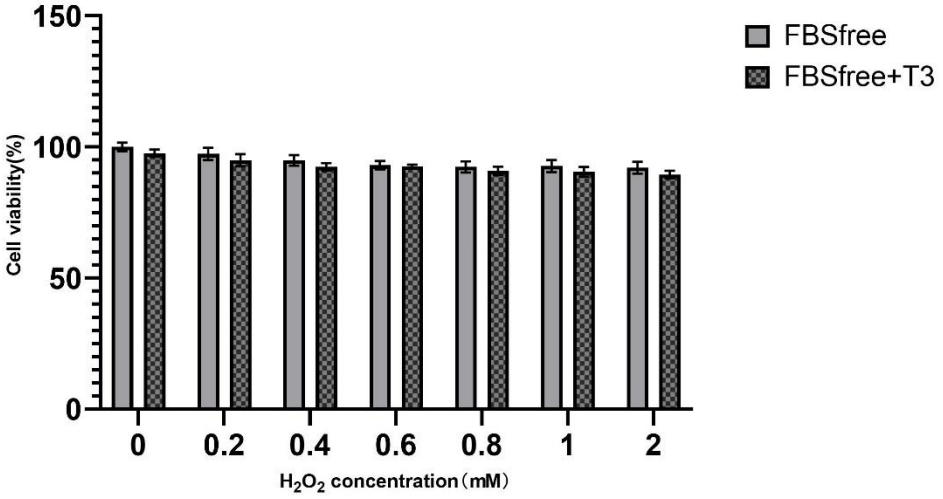


Figure 5. 7 **The effect of oxidative stress followed with 24h or 48h Hormone-free medium (HFM) +/-T3 treatment in C2C12 cells.**

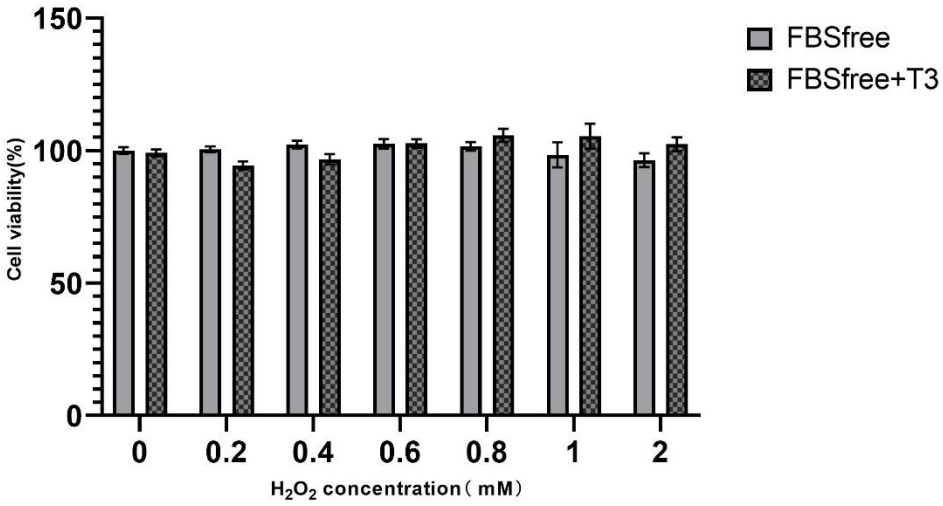
(a,b) Change in cell viability in C2C12 cells incubated with either HFM or HFM+T3 medium. Cell viability was calculated using non-oxidative stress, non T3 treated cells. (c) C2C12 48h incubation with hormone-free medium. All images were taken at 10x magnification. All data are expressed as the mean± SEM, n=6 for each treatment group. Means with the same letter are not significantly different ($p>0.05$, Bonferroni), whereas those with different letters are significantly different ($p<0.05$, Bonferroni).

In contrast to C2C12, in our experience, HK2 Cells have a higher tolerance to oxidative stress; thus, a higher concentration of H_2O_2 was used in this cell line experiment. No interaction of H_2O_2 and T3 treatment was observed ($p=0.969$ T3 x H_2O_2 24h; $p=0.166$ T3 x H_2O_2 48h); the viability of HK2 cells was mostly unchanged in the presence or absence of H_2O_2, at both 24 and 48 h ($p=0.193$ 24h; $p=0.316$ 48h). In addition, T3 treatment did not significantly affect the viability of HK2 cells ($p=0.195$ 24h T3 treatment; $p=0.354$ 48h with T3 treatment). The image of HK2 shows no visual changes in the cell structure (Figure 5.8.C).

a.HK2 24h



b.HK2 48h



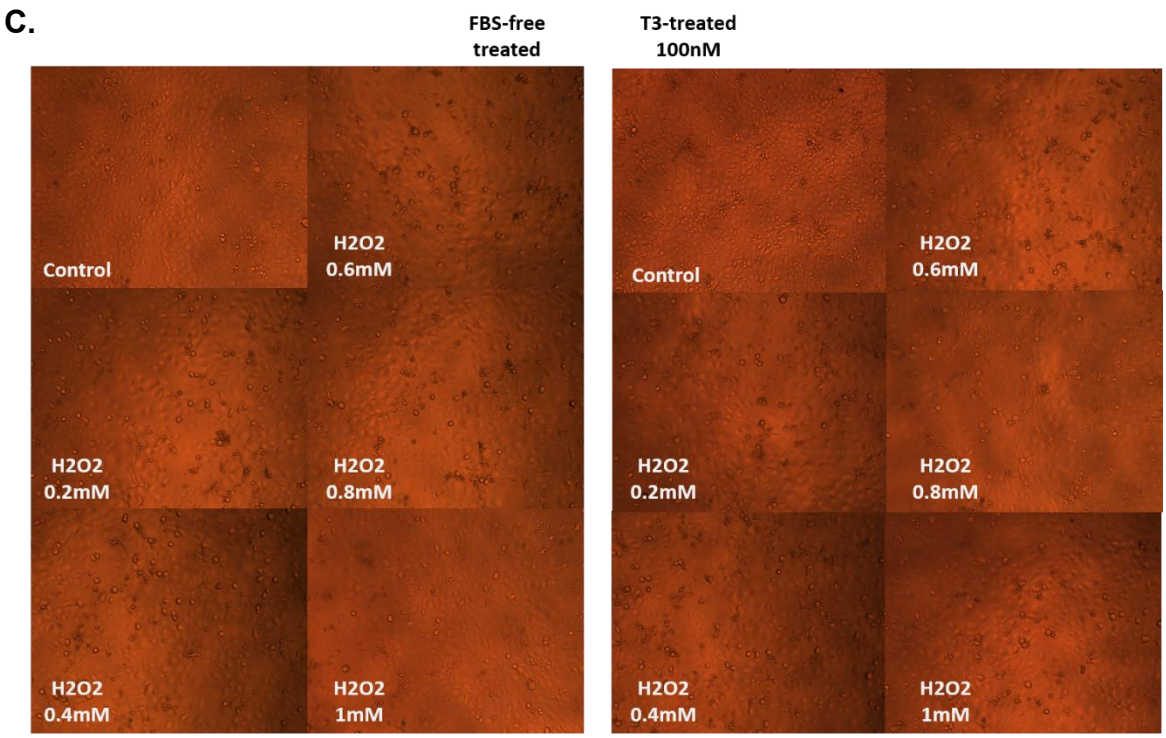
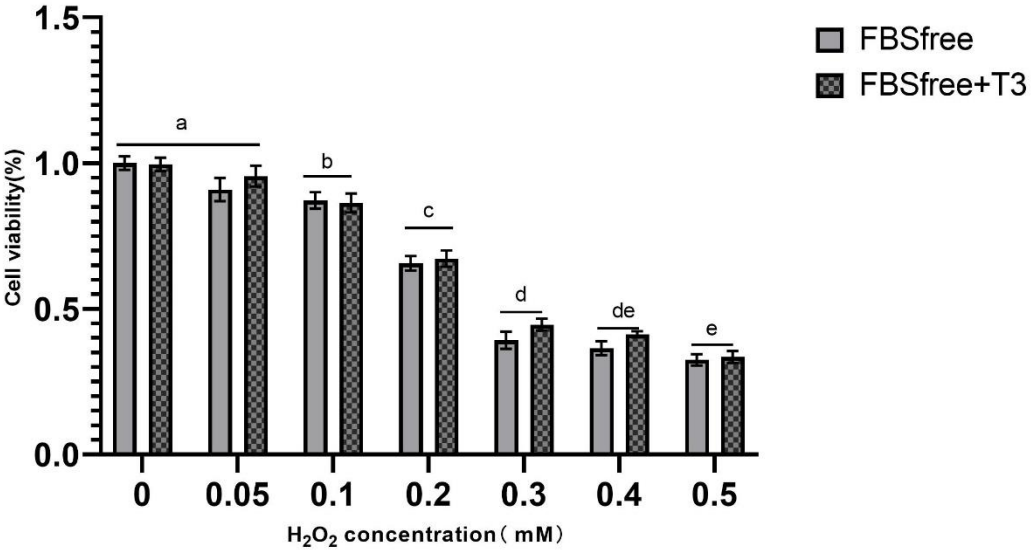


Figure 5. 8 The effect of oxidative stress followed with 24h or 48h Serum-free medium (SFM) +/-T3 treatment in HK2 cells.

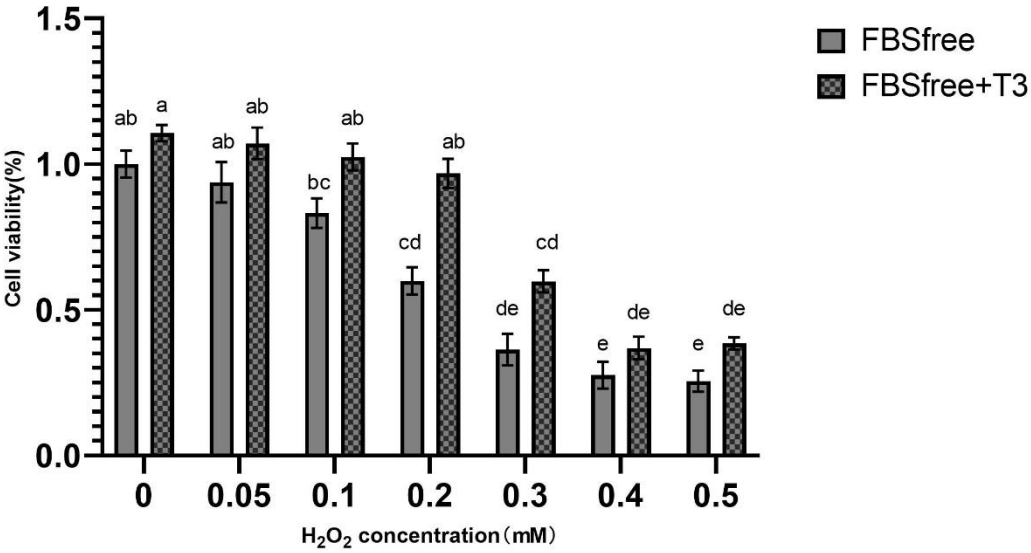
(a,b) Change in cell viability in HK2 cells incubated with either SFM or SFM+T3 medium. Cell viability was calculated using non-oxidative stress, non T3 treated cells. (c) HK2 48h incubation with hormone-free medium. All images were taken at 10x magnification. All data are expressed as the mean± SEM, n=6 for each treatment group. Means with the same letter are not significantly different ($p>0.05$, Post hoc Bonferroni), whereas those with different letters are significantly different ($p<0.05$, Post hoc Bonferroni).

After 24 h of T3 incubation following 2 h of H_2O_2 induced cell stress, no interaction between T3 treatment and oxidative stress was observed ($p=0.859$ T3 treatment × H_2O_2 level); and no significant change in cell viability was observed after T3 treatment ($p=0.485$). However, the viability of HepG2 cells significantly decreased with dose-related H_2O_2 levels ($p<0.001$). Post hoc Bonferroni test indicated that 0.1mM H_2O_2 significantly decreased HepG2 cell viability. After 48 h of T3 incubation, significant interaction between T3 treatment and H_2O_2 was observed ($p<0.001$, T3 treatment × H_2O_2 level) Post hoc Bonferroni test indicated that at 0.2mM H_2O_2 stress, the HepG2 cell viability between cells incubated with and without T3 was significantly different (Figure 5.9 b).

a.HepG2 24h



b.HepG2 48h



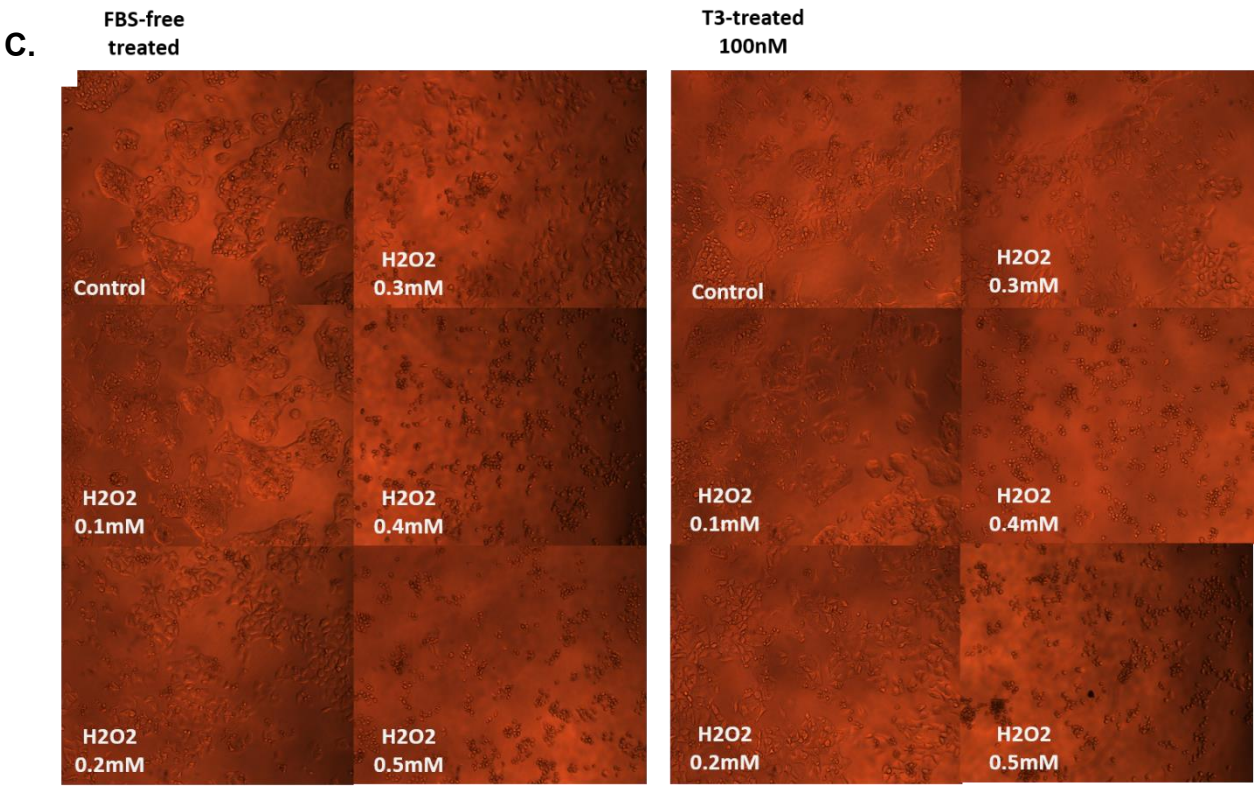


Figure 5. 9 **The effect of oxidative stress followed with 24h or 48h Hormone-free medium (HFM) +/-T3 treatment in HepG2 cells.**

(a,b) Change in cell viability in HepG2 cells incubated with either SFM or SFM+T3 medium. Cell viability was calculated using non-oxidative stress, non T3 treated cells. (c) HepG2 48h incubation with hormone-free medium. All images were taken at 10x magnification. All data are expressed as the mean± SEM, n=6 for each treatment group. Means with the same letter are not significantly different ($p>0.05$, Bonferroni), whereas those with different letters are significantly different ($p<0.05$, Bonferroni).

5.3.5 Effect of T3 treatment on target cell line HEK293 under different concentration of H2O2 induced oxidative stress.

As shown in the earlier section, the TH target cell line HK2 was not affected by 2 hours of H_2O_2 induced oxidative stress. Therefore, we used another from the same target tissue, HEK293, to investigate whether T3 has a protective effect against H_2O_2 challenge in HEK293 cells. First, we need to determine whether HEK293 is able to survive with the serum deprivation, to achieve this, HEK293 been cultured in either HFM or SFM for 48 hours as shown in figure

5.10.A. The viability of HEK293 cells cultured in SFM decreased by approximately 70% compared to that of cells cultured in HFM. Hence, the HFM was used in the next step.

As shown in Figure 5.10. B&C, the analysis performed with two-way ANOVA, indicated that no interaction between T3 treatment and different concentrations of H_2O_2 found ($p=0.629$ T3 treatment × H_2O_2 level).However, at 24 h of T3 incubation time, the viability of HEK293 cells was significantly decreased with a dose-related H_2O_2 concentration ($p<0.001$). Post hoc Bonferroni test indicated that 0.05mM H_2O_2 significantly decreased HEK293 cell viability. No statistically significant difference was observed in the effect of T3 treatment ($p= 0.0835$). Similarly, after 48 h of T3 incubation following 2h of H_2O_2 stress, no T3 and H_2O_2 interaction was found ($p=0.9425$ T3 treatment × H_2O_2 level); a dose-dependent decrease in cell viability was observed with H_2O_2 levels ($p<0.001$). Post hoc Bonferroni test indicated that 0.05mM H_2O_2 significantly decreased HEK293 cell viability. Additionally, T3 treatment did not significantly affect the viability of HEK293 ($p=0.832$ T3 treatment;).

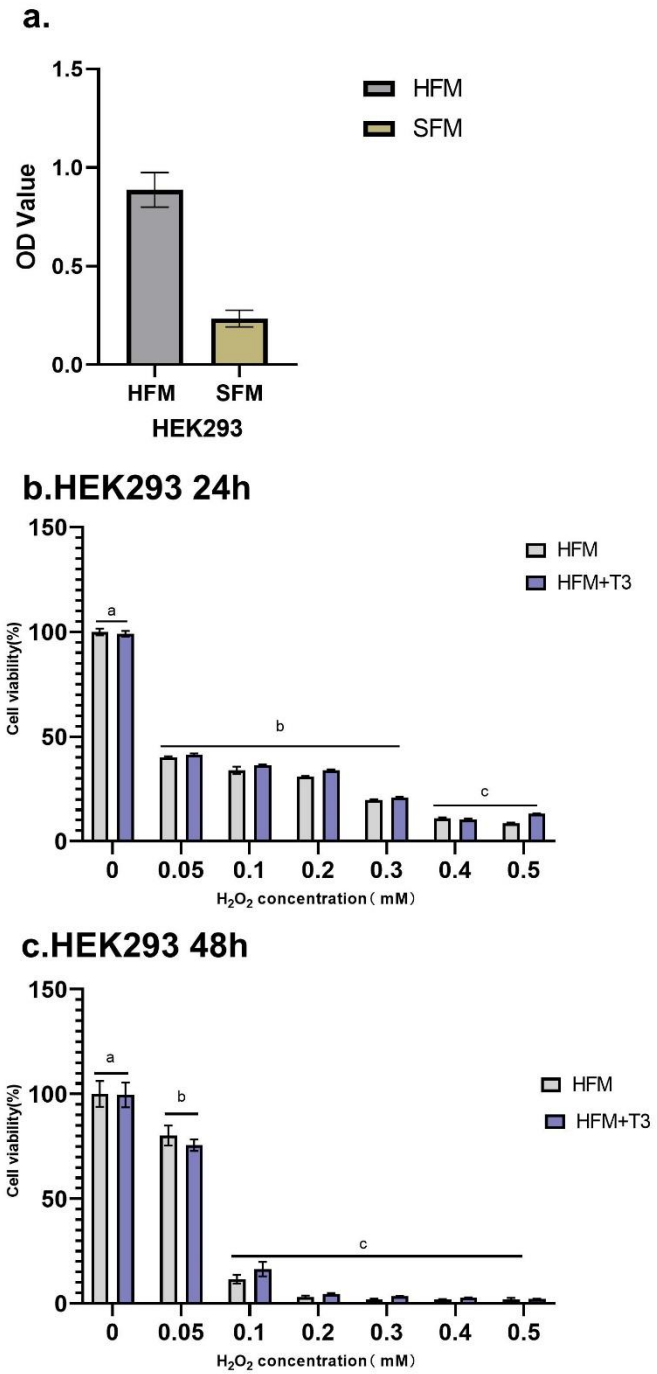


Figure 5. 10 The effect of oxidative stress followed with 24h or 48h Hormone-free medium (HFM) +/-T3 treatment in HEK293 cells.

(a,b) Change in cell viability in HEK293 cells incubated with either HFM or HFM+T3 medium. Cell viability was calculated using non-oxidative stress, non T3 treated cells All data are expressed as the mean± SEM, n=6 for each treatment group. Means with the same letter are not significantly different ($p>0.05$, Bonferroni), whereas those with different letters are significantly different ($p<0.05$, Bonferroni).

5.4 Discussion

The purpose of this chapter is twofold: first, to assess the sensitivity of in vitro cellular models of thyroid hormone target organs to thyroid hormone stimulation by testing the cell growth rate; second, to investigate whether thyroid hormones can confer protection against oxidative damage in in vitro cellular models of different target organs.

T3 increase the cell proliferation rate after 24h

First, we tested the effect of TH (T3 was used in the experiment) on the proliferation rate of different cell lines. All cell lines were cultured in hormone-free media (HFM) for 24 h before being cultured in either HFM or HFM with 100nM T3 (HFM+T3) for another 24 h, 48 h in total. A control group of cells cultured in the control medium was also performed at the same time spent of 48 hours.

In two out of the three cell lines tested, HK2 and C2C12, at the first 24 h of observation, there was a significant decrease in the cell number count in the HFM group compared to the control group. Although the result for HepG2 cells did not show statistical significance ($p=0.076$), the result indicated a decrease in the number of cells cultured with HFM. This was foreseeable for treatment for 24 h because the cells were cultured in HFM for hormone starvation. At 48 hours time point, which is 24 hours after T3 treatment, the cell count in the HFM+T3 group in both HK2 and HepG2 was significantly increased ($p<0.05$) compared to HFM treated group, C2C12 been treated with 100nM T3 shown a tendency of increase with no statistical significance($p=0.052$).

Our result indicates that adding 100nM T3 treatment hormone-free media increases the cell number in 24 hours compared to the cell cultured with only hormone-free media. This is similar to studies showing that T3 promotes the proliferation of MCF-7 and T47-D cell lines (Hall et al., 2008), CAKI-1 and CAKI-2 (Poplawski and Nauman, 2008), and LNCaP cells(Tsui et al., 2008). Flow cytometric analysis performed by Barrera-Hernandez et al. (1999) indicated that T3 stimulates cell growth by shortening the G1 phase of the cell cycle and promoting the progression to the S and G2/M phases.

However, in the study done by Poplawski and Nauman (2008) investigated the role of T3 in regulating the proliferation rate of HK2 cells and two CAKI cell lines. Their result was the opposite of our finding, showing that the proliferation rate of HK2 cells was inhibited by 100nM T3 treatment.

The reason for this difference could be certain factors of their protocol, which differ from ours. To begin with, all three cell lines were pre-starved in serum-free medium for 24 h before adding T3 treatment back into the SFM medium. For comparison, we simply starved cells in hormone-free media for 24 h and counted the cell number at both the starvation stage and 24 h of T3 treatment. In Poplawski's protocol, Keratinocyte SFM was used as the growth medium for HK2 cells. In our experiment, we used DMEM (D6546) as the growth medium. Evidence from other studies indicates that HK2 can grow in different cell culture media, including DMEM/F12 (Valdés et al., 2020), RPMI (Zhang et al., 2017) and DMEM (Du et al., 2017, Lee et al., 2017). It is possible that with different growth media, HK2 cells would react differently. Consequently, the characteristics of the cell lines might differ in different media.

In addition, it is worth noting that DMEM is often used with higher glucose levels (4500 mg/L) than KSFM (5.8mM or mmol/L = 1045 mg/L). However, the research done by Wei et al. (2019) increased the glucose level in KSFM to match the glucose level in DMEM and did not change the amount of HK2 cells. Hence, the reason behind our result differs from that of Peplowski's might be the presence of other chemicals within the culture media. However, there is insufficient evidence regarding the formation of the KSFM medium on official websites. To use the optimal characteristics of the cell line, it would be better if this experiment was performed with KSFM medium for comparison.

Changes made between two protocol.

In the MTT assay, the yellow-coloured tetrazolium salt MTT is reduced to purple-coloured formazan in metabolically active cells, particularly by mitochondrial enzymes. It was then dissolved in DMSO during the process of extracting the purple-coloured formazan from the living cells. The MTT protocol is relatively easy and effective. However, solubilisation of formazan by DMSO can result in toxicity to cells, negatively impacting cell viability and complicating data interpretation. CCK-8, on the other hand, using WSTs (water-soluble tetrazolium salts), WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) to be exact. Cellular dehydrogenases can reduce it to generate a water-soluble form of formazan. Additionally, the protocol is one step easier than the MTT assay, and has a higher detection sensitivity and lower cytotoxicity than MTT(Jiao et al., 2015). Hence, we chose the CCK-8 assay to conduct the cell viability assay. We aimed to determine whether thyroid hormones(T3) can protect against oxidative damage in *in vitro* cell models.

In the first protocol, oxidative stress was induced in the presence of T3 in an *in vitro* model of three cell lines: HK2, HepG2, and C2C12. Initially, we assessed whether the protective effect of T3 protects cell from the oxidative

damage induced by H_2O_2. To achieve this, we added H_2O_2 to the culture medium HFM or HFM+T3 for 24h before rince the cells and then culturing the cells with HFM or HFM+T3 for another 24h to prolong T3 stimulation before measuring cell viability. H_2O_2 is a commonly used approach to induce oxidative stress in experimental models and is widely used as a cell damage agent. H_2O_2 levels greater than 50uM is usually considered to be cytotoxic to the majority of organisms(Halliwell et al., 2000), we examined a wide range of H2O2 concentrations in HFM or HFM+T3 media to test the protective effect of T3.

The results showed no significant effect of T3 treatment increasing the cell viability in all the three cell lines tested. This might be due to the concentration of H_2O_2 and the duration of exposure to oxidative stress; initially, the duration of exposure was 24 h, which was an interpretation of cells chronically exposed to oxidative stress. As a result, the experimental protocol we designed and used in this study for thyroid hormone treatment may not effectively protect the tested cell lines from oxidative stress generated by H_2O_2.

Additionally, the culture serum we used in this experiment may also have affected the viability results. Normal serum contains a diverse mixture of growth factors, hormones, cytokines, and other biologically active compounds that support cell proliferation and survival (Yao and Asayama, 2017). Many of these compounds function as antioxidants that can provide a certain level of protection against oxidative stress. Hormone-free serum, on the other hand, is charcoal-stripped serum processed to remove endogenous hormones, growth factors, and other bioactive molecules.

Although the effect of hormones has been minimised due to the process of charcoal removing nonpolar molecules, hormone-free serum still obtains nutrients in the serum (salts, glucose, and amino acids). Therefore, to minimise the effects of hormone-free serum, serum-free medium was added as an additional treatment. In addition, we modified the protocol to separate the oxidative stress step from the T3 incubation step (personal advice from P Rose). The new protocol was adapted from the study by Chen et al. (2016), in which cells were serum-starved overnight before H_2O_2-induced oxidative stress was introduced for 2 h. The cells were then incubated with T3 for 48 h before viability was assessed using the CCK8 assay.

The results from the second protocol varied among the different tested cell lines.

We observed no significant impact of oxidative damage on HK2 cell line. Compared to all other cell lines tested so far, the H_2O_2 concentration used on HK2 was four times higher, indicating that HK2 has a higher tolerance level to H_2O_2-induced oxidative stress. In contrast, both C2C12 and HEK293 cells could not be rescued by T3 treatment because of H_2O_2-induced oxidative damage. Moreover, these two cell lines could not survive without serum in the culture medium. HepG2, a cell line derived from the liver tissue of a male hepatocellular carcinoma, showed a dose-dependent response to oxidative damage induced by H_2O_2, and the viability of HepG2 cells increased after 48 hours of 100nM T3 treatment.

There are several reasons for this result. The first is tissue- and cell line-specific factors. Compared to kidney and muscle tissue, liver tissue, which HepG2 originated, is highly sensitive to thyroid hormone regulation due to its crucial role in thyroid hormone metabolism. The liver is a major target for thyroid hormones, which play a vital role in regulating body weight, lipogenesis, lipid metabolism, and insulin resistance (Sinha et al., 2014). In addition, a study by Darby et al. (1991) indicated that HepG2 contains specific high-affinity binding sites for T3, which are approximately over 1000 binding sites per cell.

Despite no change in cell viability after T3 treatment, the H_2O_2 concentration used on HK2 was four times higher than that of the other three cell lines tested, indicating that HK2 has a higher tolerance level to H_2O_2-induced oxidative stress. Studies have shown that TH(T3) stimulates the absorption of Na in the renal epithelial cell line (Cano et al., 1999), as well as increases renal blood flow and reabsorption of electrolytes (Capasso et al., 1987).

The effects of TH on skeletal muscle can either increase muscle protein turnover, leading to muscle wasting, or TH can affect muscle contractility and thermogenesis, which can influence metabolic rate (Bloise et al., 2018). Although C2C12 is a skeletal muscle cell line, since the treatment was performed without differentiation, the physiology could be largely different compared to the majority of studies that used C2C12 myotubes. In their undifferentiated state, C2C12 cells resemble myoblasts which are the precursor cells that give rise to muscle cells. Additionally study by Robinson et al. (2019) indicate that undifferentiated C2C12 has significantly lower H_2O_2 emission compared to Rat L6.Suggesting a naturally low oxidative stress level, which might be the reason in our result indicating a lower oxidative stress resistance.

Second, TH transport proteins, thyroid hormone receptors, and deiodinases (D1, D2, and D3), which facilitate TH uptake and transport, vary between cell lines from different tissues, and are also important factors that determine the effect of TH(Orozco et al., 2012). The intracellular availability of TH is determined by its transport across the plasma membrane by TH transporters and the local activation or inactivation of T4 and T3, which is achieved by TH receptors and deiodinases (Figure 5.1).

The response of a cell or tissue to T3 depends on the expression of TH transporters and the presence of TRs, which can vary depending on the tissue type. Among the different isoforms of thyroid hormone receptors, TRβ is mainly expressed in the liver and kidney, whereas TRα is more commonly expressed in the cardiovascular system and muscle tissues (Sinha et al., 2014, Milanesi et al., 2016).

Most tissues are affected by circulating T3 levels, as they affect TR occupancy, gene expression, and downstream biological effects. In vivo-ly, as long as TH transmembrane transporters are available, T3 from plasma enters cells at levels that occupy half of the TR pool. As mentioned previously, TR has a higher affinity for T3 than T4, and in liver and kidney cells, approximately half of the TR pool is occupied by T3 derived from the circulation. On the other hand, a previous investigation by Larsen and Zavacki (2012) found that skeletal muscle primarily depends on the endogenous conversion of T4 to T3, rather than on serum T3 levels. Another study by Lee et al. (2017) showed that a significant change can be found in mouse muscle at the level of deiodinase D2 and TTR. Their results indicate the active conversion of TTR-bound T4 to T3 by D2. In addition, they showed that TTR expression gradually increased during the proliferation of C2C12 myoblasts.

Hence, the argument could be made that when considering the testing of multiple cell lines and their relationship with thyroid hormones, simplifying the process by using only T3 might not be the most appropriate.

Aside from the HepG2 cell line, cell viability levels that had been oxidatively stressed with H_2O_2 were not increased by T3 in any of the three cell lines tested. In addition, in all the cell lines tested, C2C12 was shown to be most sensitive to H_2O_2-induced oxidative stress, followed by Hek293, then HepG2, and HK2, which had the highest tolerance level. A previous study done by Ryan et al. (1994) observed that H_2O_2 significantly reduced the proliferation rate of HK2 with a concentration of 17mM.The resistance of HK2 to the H_2O_2 concentration we were using could be due to the strong defence it acquired compared to other cell types we used in this chapter.

Furthermore, we noticed that when only comparing groups with no H_2O_2 and with or without T3 treatment in the cell viability assay in 96-well plates, there was no significant difference regarding the involvement of T3. This is the opposite of the results we had that determined cell numbers through cell counting in the 6-well plates. Several factors may have accounted for this discrepancy. First, the methods employed to measure cell viability and count cells differ fundamentally in their approaches. For the viability assay, we used a 96-well plate, which relies on metabolic activity and reduction of a dye indicator. In the cell counting assay, 6-well plates were used, and direct quantification of the total cell population was performed. Second, the microenvironment within the 96-well plates, including factors such as cell density, nutrient availability, and growth conditions, can differ significantly from those in 6-well plates, which may impact cellular responses and viability. Additionally, variations in the sensitivity and specificity of the two assays may have contributed to the observed differences.

Therefore, it is crucial to take into account these methodological differences and environmental factors when interpreting the conflicting results obtained from these two approaches.

In conclusion, in the current study, we attempted to determine the sensitivity of in vitro cellular models of thyroid hormone target organs to thyroid hormone (T3) stimulation, measured the cell growth rate, and investigated whether thyroid hormones could provide protection and recovery against extracellular oxidative stress in in vitro cellular models of target organs. We tested multiple cell lines and that introduced a complex layer of variability into our experimental findings. Our experiments have yielded some valuable results that have shown the sensitivity of different cell lines to H_2O_2-induced oxidative stress, as well as their different responses to T3. Nevertheless, it is crucial to acknowledge that the different cellular responses across different cell lines may be attributed to the presence of diverse TH transporters, receptors, and deiodinases within these cell types, which have not yet been examined. Hence, based on the limited available data, it would be premature to draw definitive conclusions. Thus far, our findings should be considered initial observations that require further investigation.

To gain a comprehensive understanding of the cellular processes involving TH, it is crucial to investigate the expression profiles of different transmembrane transporters, deiodinases, and related molecular components in various cell lines. In addition, we will include T4 in future studies. By conducting comprehensive investigations, we can create a solid and robust foundation for understanding the TH-related mechanisms.

Chapter 6 Discussion, Limitation and Conclusion

Iodine is a crucial nutrient for human development, growth, and metabolism, as it is the key component for the synthesis of the thyroid hormones T3 and T4 by the thyroid gland (Kopp and Solis-S, 2009, Braverman and Cooper, 2013). Thyroid hormones (THs) are crucial regulators of metabolism, growth, and development across the human lifespan, from the prenatal stage to adulthood. They play a vital role in almost every organ system, contributing to the maintenance of the homeostatic balance necessary for overall health and well-being(Song et al., 2011, Brent, 2012, Cheng et al., 2010). The effects of THs are relevant in humans at every stage of development, and their importance cannot be overstated.

The ideal dietary iodine intake recommended by the WHO is 150μg per day (Table 1.1) which increases to 200-250μg per day in pregnancy due to its crucial role in brain and neurological development(Williams, 2008). Fish, milk and dairy products, and eggs are considered the primary sources of dietary iodine(Dahl et al., 2004, Nerhus et al., 2018). It is commonly thought that iodine insufficiency is limited to developing countries and areas with iodine-poor soils. However, recent research has revealed that iodine deficiency is also prevalent in the UK and other European countries, particularly in susceptible groups, such as women of reproductive age and children(Vanderpump et al., 2011, Mian et al., 2009, Zimmermann and Andersson, 2012b). Given various factors, such as modern lifestyle changes, diverse dietary choices, and farming practices, it is reasonable to assume a fluctuation in iodine intake. Despite the common occurrence of these fluctuations, no existing studies have focused on the fluctuations in iodine intake and their subsequent impact on thyroid health.

Thus, our study aimed to investigate the impact of iodine intake fluctuations, and we are keen to study the situation from multiple perspectives, both public health perspectives and experimental research.

6.1 Analysing Iodine Status and Fluctuations in the UK: Insights from the UIC and Primary Dietary Sources.

In this study, we observed that from the newest NDNS report, the median UIC from all age groups in both genders reached the adequate iodine intake for the population (Table 3.5). For females in the reproductive age group (19-45 years), the UIC was at the borderline level of the adequate at 100 µg/L Studies have consistently shown that women of reproductive age exhibit low median UIC, indicating iodine deficiency(Mian et al., 2009, Bath et al., 2014, Panth et al., 2019) The main contributors to iodine intake were milk and dairy products in all age and gender groups, ranging from 34.28% to 52.19% of the total iodine intake. Several studies reported the predominant place of milk and dairy product as iodine contributor in industrialized countries which some of them does not have a USI such as UK (Pearce et al., 2004, Dahl et al., 2004, Dineva et al., 2021). Based on the fluctuation rating score we created (Table 3.4), the main food source that contributed to the fluctuation in all age and gender groups was identified as fish and fish products (Table 3.10 to 3.17).

In addition, a positive correlation was found between the mean iodine intake and the fluctuation rating factor (measured by the standard deviation, SD) (Figure 3.3) and the mean iodine intake with UIC (Figure 3.2). This inversely proves that there is a potential correlation between the UIC and the fluctuation factor (Figure 3.1). A weak correlation was observed between the two age groups. This may be weakened by the long period between the food diary and urine sample collection.

6.1.1 Limitation of using the NDNS as a population database

As highlighted in our previous chapter discussion, the NDNS provides comprehensive nationwide data, including detailed nutritional information. However, considering the traceability of iodine through urinary analysis, the timing of sample collection encompassing both urine and food diaries is crucial. The study conducted by Katagiri et al. (2016) underscores the significance of synchronisation between dietary records and urine sample collections. They found that a 4-day (non-consecutive) food diary paired with

a 24-hour urine collection yielded a stronger correlation in iodine intake assessments when the urine sample was collected concurrently with the dietary record. In contrast, there was a three-month gap between the food diary dispatch – face-to-face interview (first time and potential urine sample collection)–food diary instruction (second interview)– food diary collection (third interview and potential urine sample collection) periods. The uncertainty of urine sample collection is a limitation of NDNS. Additionally, the NDNS method of gathering a single spot urine sample from each participant, starting at the age of four, has limitations in accurately describing the prevalence of iodine status owing to its snapshot nature (Zimmermann, 2008, Zimmermann, 2009, Andersen et al., 2008). The justification for using spot urine samples in large-scale studies, such as the NDNS, is often based on practical considerations, such as cost, participant compliance, and logistical feasibility.

Furthermore, the use of creatinine to adjust UIC measurements is beneficial and common (Garçon et al., 2007, Montenegro-Bethancourt et al., 2015, Ji et al., 2020). However, the NDNS did not collect data on urinary creatinine, although blood creatinine levels were recorded for years 9 and 10. This omission prevented a more accurate understanding of the iodine status of the UK population, as urinary creatinine level would have provided a more detailed perspective. Speaking of blood sample, in the previous NDNS, through year 1-5, the serum TSH, fT3 and fT4 was collected aside from the urine sample during those survey years was solely for sodium monitoring purposes(Terry et al., 2016).

6.2 Building the fluctuation in vitro model.

In this study, we successfully designed an in vitro model that mimics fluctuating iodine intake. There has been little to no assessment of the impact of fluctuating iodide exposure using in vitro systems. Nthy-ori 3-1 human thyroid follicular cell line was used in this study. As shown in Figure 4.4, the fluctuation model sample was collected at two time points: fluctuation down (iodide absence) and up (iodide reintroduction). We observed that TSHR was significantly influenced by both TSH and iodide, consistent with the findings of previous studies (Huang et al., 2016, Kleinau et al., 2010, Zoenen et al., 2012,

Kleinau et al., 2017). The binding of TSH to TSHR activates the signal pathway that regulate many thyroid hormone synthesis related gene expression including PAX8, TPO, PDS, DUOX2 and NIS. Similar to what previous study shown (D'Agostino et al., 2014, Kambe and Seo, 1997, Pellizzari et al., 2000, Huang et al., 2016, Jing and Zhang, 2022).

Additionally, as mentioned in the Discussion section, the expression differences of NIS mRNA and protein (Figure 4.8). It can possibly be explained by many other factors aside from TSH, such as translation delay post transcriptional modifications (Riedel et al., 2001) and experimental practice issues. A study by Serrano-Nascimento et al. (2010) suggested that the abundance of NIS mRNA was significantly decreased after treatment with excess NaI, resulting in the speculation of transcript stability. This can influence stability and translation efficiency (Munroe and Jacobson, 1990, Sachs, 1993). Moreover, ER stress can also trigger delayed translation (Cheng et al., 2016), an indicator of ER stress, GRP78, which also showed an increase in the fluctuation phase of the study.

Furthermore, an increase in the autophagy indicator LC3B was also found in the fluctuation down phases, which is an interesting discovery as it indicates that during the cell was in the iodine starvation state (i.e. it was cultured in an iodine-sufficient or excess environment), the thyroid cell model tried to undergo autophagy in order to maintain homeostasis(Rubinsztein et al., 2005, Yun et al., 2020). This also hypothetically explains the findings of the ROS level assessment. Thyroid-stimulating hormone (TSH)-stimulated thyroid cells are perpetually subjected to the effects of ROS (Kim et al., 2001). The ROS result was also affected by the antioxidant effect of iodide in both of the fluctuation model (Figure 4.13) as well as the continuous iodide incubation model (Figure 4.16)

6.2.1 Limitations

Although we acknowledge the designed fluctuation model in this study, has been a successful attempt, it is also important to note that there are still many

genes and proteins related to thyroid hormone production that have not been thoroughly tested or explored. One example is TG, which plays a crucial role in TH production (Citterio et al., 2019). Thyroglobulin serves as a scaffold for thyroid hormone synthesis and storage, making its investigation vital for a comprehensive understanding of TH biosynthesis and regulation. However, due to the nature of in vitro cell study, as study reported before, it could be hard to get the ideal result since the monolayer incubation may not be able to trap TG.

Additionally, our attempts to implement a transwell model encountered challenges. This model, designed to simulate the thyroid microenvironment more accurately and allow for the study of cellular interactions and hormone transport, did not achieve the expected outcomes (section 4.3.9). The failure of this model highlights the complexity of thyroid physiology and underscores the need for refined approaches to better mimic the intricate processes occurring within the thyroid gland. Further research and development are required to overcome these hurdles and create more effective models for studying thyroid function and disease.

6.3 Thyroid hormone (T3) was able to up-regulate the proliferationrate but no protective effect with target cell lines.

As shown in section 5.3.1, the T3 after 24h of hormone starvation was able to significantly increase the proliferation rate of target cell line in our experiments, including HK2, C2C12 and HepG2. Numerous studies have shown similar results (Hall et al., 2008, Poplawski and Nauman, 2008). However, when attempting the T3 protecting against oxidative damage in in vitro cell models, the first protocol shown negative results (Figure5.5). As shown in Figure 5.3.B, the first protocol was involved with 24 hours of H_2O_2 incubation with FBS (charcoal-stripped). Although charcoal treatment has reduced the impact of hormones by removing nonpolar molecules, hormone-free serum still contains essential nutrients, such as salts, glucose, and amino acids. To minimise the effects of hormone-free serum, serum-free medium was added as an additional treatment. Furthermore, we modified the protocol to separate the oxidative stress step from the T3 incubation step (Figure

5.3.C). Aside from HepG2, no significance of T3 effect was found in all other tested cell lines. This might be tissue- and cell line-specific factors; since liver is a main target of TH (Sinha et al., 2014).

6.4 Conclusion

To conclude, iodine status gathered from NDNS indicates an adequate iodine level in the UK population, with a borderline deficiency in reproductive-age females. Milk and dairy product is the predominant source for iodine in the UK population. Fish and fish product is the most contributor to the iodine intake fluctuation. An in vitro iodine fluctuation model successfully observed the interaction between TSH and the fluctuation of iodine levels affecting thyroid hormone production-related genes, including NIS, TSHR, PAX8, DUOX2 PDS, and TPO. In addition, the expression of the ER stress marker was up-regulated with TSH but decreased after the reintroduction of iodide; the up-regulation of the autophagy indicator shows during the iodine starvation phase, thyroid cells used autophagy to maintain homeostasis.

The autophagy process during the fluctuation might also be relevant to the ROS reduction by TSH.

6.5 Future works

As mentioned in previous sections, from public health perspective, in order to better monitor the level of iodine fluctuation We suggest two methods: maintaining a four-day food diary every month for at least ten months to a year, ideally along with concurrent spot or 24-hour urine samples for UIC assessments; or keeping a detailed ten-week food diary and providing weekly urine samples.

Furthermore, since we have built the fluctuation model, however, the result we got so far was limited into one fluctuation cycle. Hence, we propose that with A) increase the fluctuation cycle number in order to conduct more comprehensive research with the effect of iodine fluctuation to TH synthesis

pathways; B) changing the fluctuation cycle into more randomized design in order to mimic the irregularity of human diet.

Chapter 7 References

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