The Effect of Hyperbilirubinaemia on Hepatic Cholesterol Metabolism in the Gunn Rat

Johara Pillay BBiomedSc

s2946061

Primary Supervisor: Associate Professor Andrew Bulmer

Secondary Supervisor: Associate Professor Eugene Du Toit

School of Medical Science

Griffith University

Submitted in fulfilment of the requirements of the degree of

Master of Medical Research

November 2018

Statement of Originality

The Effect of Hyperbilirubinaemia on Hepatic Cholesterol Metabolism in the Gunn Rat

Johara Pillay

School of Medical Science

Griffith University

This work has not previously been submitted for a degree or diploma at any university. To the best of my knowledge and belief, this dissertation contains no material previously published or written by another person except where due reference is made in the dissertation itself.

Miss Johara Pillay

Date: 20/11/2018

Master of Medical Research

Griffith University Gold Coast, Queensland, Australia

Acknowledgements

First and foremost, I would like to thank my primary supervisor, A/Prof Andrew Bulmer. Your guidance, and mentorship has been so valuable in developing my skills and attitudes towards research. Your enthusiasm for your research and the opportunities that arise from that are two traits that I will happily take away from this experience. I truly appreciate the time taken to assist in the lab, mark drafts and remain involved in the project. Your words of encouragement were very much appreciated and thank you for reminding me of the bigger picture, especially during the more challenging times.

Secondly, I would like to extend a massive thank you to Josif Vidimce for assisting me in the running of this project. Your work ethic and attitude towards getting the work done, and doing it well is something that I truly admire and will stick with me beyond this project. I would like to thank you for the constant guidance, words of encouragement, feedback and willingness to discuss the possibilities of the project. I especially thank you for your positivity and fantastic company, whether working ungodly hours in the animal house, learning new seemingly impossible techniques, which are now natural, tediously entering data and now finally seeing the bigger picture, you have made this whole experience so much more enjoyable. I wish you all the best with your completion of your PhD, you absolutely deserve the best! So, thank you.

To the Bulmer Lab Members, especially Evan Pennell and Ryan Shiels, I truly appreciate your assistance when I (sometimes frantically) needed. It has been an absolute pleasure being a part of the Vabbi Research Group and I wish everyone the best in their completion of their degrees.

To the animal house staff, especially Hayley Duncan and Allanah McMath, I'd like to say thank you for your assistance in colony management and training. It has been a pleasure to work with you.

I would like to extend thanks to our international collaborators, Prof Henkjan Verkade and Prof Karl-Heinz Wagner for the opportunity to collaborate with you on the current project. The

Verkade group especially for the analysis of samples, and explanation of concepts. Your efforts were truly appreciated.

To my fellow HDR students. You have seen all the ups and downs that comes with lab work, and writing and everything HDR, and have made this experience so enjoyable. Your constant encouragement and exciting discussions are appreciated. I wish you all the best on your journeys.

Finally, I would like to extend a very special thank you to my friends and family for your constant support and understanding when I haven't been particularly present. Your love and understanding throughout the whole experience is so appreciated. Special mention to my parents, who, despite not knowing the ins and outs of cholesterol metabolism, has shared numerous discussions, rants and celebrations with me. You are the real MVPs and I love you so much!

Statement of Contribution

Miss Johara Pillay

- Animal protocol implementation
- Animal surgery/bile duct cannulation
- Hepatic/faecal lipid extraction
- Collection of tissue (adult lipid study)
- Collection of tissue (juvenile lipid study)
- Blood and bile biochemistry analysis
- Data collection/analysis
- Colony management
- Wrote the thesis

Dr Andrew Bulmer

- Primary supervision
- Development of study design/animal ethics application
- Colony management
- Assistance in animal handling training (gavage, i.p. injection)
- Training in animal surgery/bile duct cannulation
- Assistance in data interpretation
- Assistance with thesis editing

Dr Eugene Du Toit

- Assistance with thesis editing

Mr Josif Vidimce

- Assistance in development of study design/animal ethics application
- Animal protocol implementation
- Collection of tissue (adult lipid study)
- Collection of tissue (juvenile lipid study)
- Assistance in blood/bile biochemistry analysis
- Sourcing equipment (metabolic cages, anaesthesia)

- Assistance in data interpretation
- Assistance in thesis editing

Dr HJ Verkade, Onne Rhonda

- Development of C^{13} lipid analysis protocol
- GC-MS analysis of isotopically labelled cholesterol in blood spots

Hayley Duncan and Allanah McMath

- Colony management
- Phenotyping and sexing of animals prior to weaning
- Animal maintenance

Mr Evan Pennell

- Assistance in COBAS analysis

Abstract

Cardiovascular Disease (CVD) is one of the world's leading causes of morbidity and mortality, contributing to approximately 30% of the total deaths in Australia. CVD affects the heart and its associated vasculature and affects people of all ages and socio-economic backgrounds. Hypercholesterolaemia is key risk factor associated with the onset of CVD, particularly through its direct contribution to the development of atherosclerosis. Elevation in circulating cholesterol contributes significantly to the progression of atherosclerotic plaques leading to cardiovascular events such as embolism, myocardial infarction and stroke. Recent meta analyses show that people with Gilbert's syndrome, a mild benign form of hyperbilirubinemia, experience less severe CVD and have an improved plasma lipid profile compared to normobilirubinaemic individuals. This finding has sparked research into the possible cardioprotective effects of bilirubin, which was previously only considered diagnostic test for the presence of liver dysfunction. Since then, the powerful antioxidant potential of bilirubin has been extensively investigated, and currently is considered a major mechanism contributing to the cardioprotective effect of the molecule. However, recent evidence suggests that mild hyperbilirubinemia may improve lipid status. This study aimed to determine whether hyperbilirubinemia is associated with lipid lowering effects in Gunn rats, a mutant model of chronic unconjugated hyperbilirubinaemia. By analysing endogenous cholesterol synthesis and biliary lipid secretion, amongst other parameters, this study aimed to develop a deeper understanding of impaired bilirubin excretion (i.e. UGT1A1 dysfunction) on cholesterol/lipid metabolism.

Methods: 36 age- and litter-matched 10-week-old adult hyperbilirubinaemic Gunn rats (male n=9, female n=10; homozygous) and controls (male n=8, female n=9; heterozygous) were compared in order to determine potential differences in cholesterol metabolism associated with UGT1A1 dysfunction. All rats were exposed to a 19-day protocol, where regular blood samples

were collected (day 0, 7, 19) and animals placed in metabolic cages for 5, 24 hour periods (day -1, -4, 10, 14, 18). In the final 5 days, the water supply was supplemented with a stable isotope (C^{13}) label of acetate (2% w/v), to which animals had *ad libitum* access. On the final day, the bile duct was cannulated (following appropriate anaesthesia) and bile collected for 30 minutes. Analysis of tissue including blood, liver and faeces was undertaken to determine possible differences in blood and bile biochemistry. Repeated blood samples collected in the final 5 days of the protocol were analysed using GC-MS to calculate fractional cholesterol biosynthesis. Analysis of serum samples were also completed in 30, 3-week-old *juvenile* Gunn (male n=10, female n=7; homozygous) and control (male=n=8, female n=5; heterozygous) animals to assess any differences associated with sexual maturity.

Results: Gunn rats had significantly reduced body weight (Gunn = 190.92, control = 245.9, $p<0.01$; g) and food intake (control = 20.0 ± 1.84, Gunn =16.4 ± 2.06, $p<0.001$; g). Analysis of plasma biochemistry revealed a notable reduction of lipids HDL (control = 1.36 ± 0.25, Gunn =0.20 ± 0.09, $p<0.001$; mmol/L), total cholesterol (control = 1.56 ± 0.34, Gunn = 0.60 ± 0.12, $p<0.001$; mmol/L) and an increase in plasma bile acids (control = 16.3 ± 7.34, Gunn = 41.7 ± 27.14, $p <0.05$; μmol/L) .These observations were specific to female animals only. Analysis of biliary constituents and relative bile secretion revealed significantly greater biliary cholesterol secretion in female Gunn rats, compared to female controls (females; control = 73.7 ± 40.0, Gunn 153.3 ± 27.7, $p=0.006$; mmol/L). However, no changes in hepatic cholesterol content and faecal cholesterol excretion were observed. Fractional cholesterol biosynthesis was significantly increased in female Gunn rats, however, no difference was observed in males. Analysis of juvenile serum biochemistry in Gunn rats, revealed non-significant reductions in plasma cholesterol and bile acids, but significant reduction in phospholipids (controls 150 ± 31.8, Gunn 118 ± 23.1 , $p<0.05$; mg/L), compared to controls.

***Conclusion*:** This study is the first to assess of fractional cholesterol synthesis and biliary lipid output, providing insight into cholesterol metabolism of adult Gunn rats. It was originally hypothesised that endogenous synthesis is increased in Gunn rats, compared to controls. Female Gunn rats displayed an increased rate of fractional endogenous synthesis, compared to control females. The males on the other hand, did not show any significant differences. Increased endogenous synthesis in female Gunn rats suggests reduction of circulating cholesterol might be due to alternative mechanisms, with increased circulating bile acid concentrations suggesting the upregulation of CYP7A1, redirecting cholesterol to bile acid synthesis, or the downregulation of ABCA1, reducing cholesterol efflux from the periphery. Increased biliary cholesterol secretion was observed in female Gunn rats, compared to female controls, but no difference observed in males. Sex specific effects of UGT1A1 deficiency, seen in the current study, suggest that sexual hormones are influential in the interaction between chronic hyperbilirubinaemia and cholesterol metabolism. This was not addressed in depth, in the current study. Further confirmation of this hypothesis, using stored tissue samples from this study, could implicate new roles for hyperbilirubinaemia/UGT1A1 dysfunction in perturbing lipid metabolism and protecting from CVD.

Table of Contents

List of Abbreviations:

-tg	"Transgenic"
ABCA1	ATP-binding cassette transporter
ABCB11	ATP-binding cassette transporter ABCb11
ABCB1a/b	ATP-binding cassette transporter 1 a/b
ABCB4	ATP-binding cassette transporter
ABCC2	ATP Binding Cassette Subfamily C Member 2
ABCG5/8	ATP-binding cassette transporter
ACAT	Acyl-coenzyme A:cholesterol acyltransferases
Ad Libitum	At liberty
ADP	adenosine diphosphate
Apo A/B	Apolipoprotein A/B
ATP	Adenosine Triphosphate
ATP8B1	ATPase Phospholipid Transporter 8B1
BA	Bile acids
BDT	Bilirubin Ditaurate
BMI	Body Mass index
BSDF	Bile salt dependent flow
BSEP	Bile salt export protein
BVR	Biliverdin reductase
CAD	Coronary artery disease
CB	Conjugated bilirubin
CN -1/2	Crigler-Najjar Syndrome
CVD	Cardiovascular Disease
CYP7A1/1A1/1A2	Cholesterol 7/1/1 alpha-hydroxylase 1/1/2

De Novo	"Newly"
EHBR	Esai Hyperbilirubinaemia Rat
FFA	Free Fatty Acids
FXR	Farnesoid X reception
GS	Gilbert's syndrome
HDL	High density Lipoprotein
HMGR	Human 3-hydroxy-3-methylglutaryl-CoA reductase
HO-1/2/3	Haem-oxygenase
IDL	Intermediate density lipoprotein
IHD	Ischaemic heart disease
IMT	Intima Media Thickness
In vitro	Performed or taking place in a test tube, culture dish
In vivo	Taking place in a living organism
Insig-1	Insulin-induced gene 1
LDL	Low Density lipoprotein
LDLr	LDL receptor
MDR2/Mdr2	Multidrug resistance-2 gene (HUMAN/rodent)
MIDA	Mass isotopomer distribution analysis
MiR33A	Micro RNA 33A
MK	Mevalonate Kinase
MRP2/Mrp2	Multidrug resistance-associated protein 2
NO	Nitric Oxide
NPC1L1	Niemann- pick like protein
OATP	Organic anion transport protein
oxLDL	Oxidised LDL
PBS	Phosphate Buffered Saline

PFIC	Progressive familial intrahepatic cholestasis
PMK	Phosphomevalonate Kinase
PPAR	Peroxisome proliferator-activated receptors
PS	Phosphatidylcholine
RCT	Reverse cholesterol Transport
ROS	Reactive oxygen species
SR-BI	Scavenger receptor class B type 1
SREBP-2	Sterol regulatory element-binding proteins
TG	Triglycerides
TICE	Transintestinal Cholesterol Excretion
TR-	Transporter deficient
UCB	Unconjugated bilirubin
UGT1A1	UDP-glucuronosyltransferase 1-1
VLDL	Very low-density lipoprotein
VSMC	Vascular Smooth Muscle Cells

List of Figures:

List of Tables:

Introduction

Cardiovascular disease (CVD) is one of the leading causes of disease morbidity and mortality, contributing to approximately 30% of deaths in Australia. CVD describes diseases of the heart and its associated vasculature, including ischaemic heart disease (IHD), coronary artery disease (CAD) and stroke. These diseases affect all demographics, irrespective of ethnicity and socio-economic status [1]. Australian health statistics have identified ischaemic heart disease as the leading cause of death for over a decade [2, 3]. It is, therefore imperative that research concerning CVD is undertaken, to further understand the progression of disease, and develop effective methods of treatment.

Statins are currently used to reduce circulating LDL-cholesterol concentrations and exert their effect by inhibiting the rate limiting enzyme of the cholesterol biosynthesis pathway, HMG-CoA reductase (HMGR) [4]. Statins are effective in reducing the 5-year incidence of major coronary events, and stroke, in proportion to its reduction of serum low density lipoprotein (LDL) [5]. Although this treatment option is effective in reducing cholesterol, adverse side effects including muscle damage [6] and complications surrounding the central nervous system [7] , can compromise the overall benefit of the drug. Therefore, alternative methods of reducing circulating cholesterol are needed, to supplement statin therapy, and provide a safer treatment for hypercholesterolaemia.

Early studies have noted altered lipid profiles in individuals with Gilbert's syndrome (GS), a life-long condition of mild, benign hyperbilirubinaemia. Individuals with GS display reduced total, LDL cholesterol and Body Mass Index (BMI) [8]. Aside from possessing a mild elevation in circulating unconjugated bilirubin (>17.1 μM), leading to occasional jaundice, no adverse effect has been reported in these individuals. Indeed, some studies infer greater longevity in chronically hyperbilirubinaemic individuals [9], suggesting this syndrome might confer beneficial effects. The risk reduction concerning CVD and age related death in GS [10],

highlights the potential for novel and highly effective treatments for CVD, utilising bilirubin metabolism.

This study aimed to provide insight into the lipid status of the hyperbilirubinaemic Gunn rat. The focus of this study was to identify potential areas at which bilirubin/UGT1A1 inactivity mediates lipid lowering effects, focusing on the cholesterol biosynthetic pathway. The assessment of endogenous cholesterol biosynthesis, plasma lipid status and biliary lipid secretion, provide this insight, relative to male and female heterozygote, normobilirubinaemic animals. Additional variables including bodyweight, hepatic cholesterol content and faecal cholesterol content/excretion complemented the above parameters and allow for discussion with existing literature in the field. Understanding the mechanism behind the favourable lipid profile observed in Gilbert's syndrome, may represent an important first step to the development of a new and novel treatment option for hyperlipidaemia.

1.0 Literature review

1.1 Global burden of disease

Globally, cardiovascular disease is a primary contributor to all disease morbidity and mortality, accounting for ~31% of all global deaths in 2016 [11]. Global statistics reveal notable decreases in the mortality rates of CVD in the developed world, compared to that of developing countries, which now account for less than one quarter of total CVD associated deaths [12].

Australian CVD statistics are similar to global trends, with CVD accounting for over 20% of deaths in Australia [2]. Earlier reports of death and morbidity, released by the Australian Bureau of Statistics, reveal ischaemic heart disease (IHD) as the leading cause of death amongst men, and women, contributing to 23,813 deaths in 2008 [2]. In just under a decade following, the number of deaths associated with IHD has declined to 18,590 in 2017 [2]. This steady reduction reflects the positive effect of modern medical practice, however, does not diminish the importance of ongoing research.

According to the Australian Institute of Health and Welfare, the health burden associated with CVD spans beyond death statistics, where CVD has been the cause of <1.1 million hospitalisations, contributing to 11% of total hospital admissions in Australia alone. This accounts for the highest cost burden of any other disease group, with an estimated total cost of $7.7 billion dollars, or 10.4% of total allocated disease expenditure in 2012 [11] (Figure 1.1).

Figure 1.1 *Allocated healthcare expenditure in Australia, by disease group and area of expenditure [11]*

It is possible that the cost of preventative measures, including pharmaceuticals and health appointments, could be contributing to the increased prevalence of CVD in low socioeconomic areas. This demographic displays the highest rate of hospitalisations and death resulting from CVD in Australia [1, 13]. Despite the reduction of CVD related morbidity and mortality in the past decade [3], the annual expenditure for treatment and prevention of CVD is expected to rise, as further preventative measures are adopted, and population size increases [4].

1.1.1 Hypercholesterolaemia as a risk factor for CVD

In 2004, Yusuf at al. published the results of the INTERHEART study, which examined more than 27000 cases and controls from 52 countries [14], identifying nine modifiable risk factors that explain more than 90% of the population attributable CVD risk. Those being: apolipoprotein B/apolipoprotein A ratio, smoking, diabetes, hypertension, abdominal obesity, psychosocial factors, fruit/vegetable consumption, physical activity and alcohol consumption. The Framingham Heart Study, later analysed by Castelli et.al [15] identified a negative relationship between high-density lipoprotein (HDL) and the onset of CVD. A positive correlation seen in total cholesterol and the incidence of coronary heart disease was observed by Keaven et al. [16] in the same Framingham study cohort. Since then, a number of studies have demonstrated a positive relationship between total serum cholesterol [17] or low-density lipoprotein (LDL) [18] and prevalence of heart disease (CHD). Bae et al. [19], observed a u-shaped correlation between serum total cholesterol concentrations and CVD mortality, in Korean adults, highlighting the risk of severe reduction in cholesterol, similarly noted in a previous study by Jacobs et al. [20]. Correlation analysis of specific lipid profile was not included in either of these studies, however, HDL cholesterol concentrations were assessed as a confounding factor. It is evident amongst the discussed studies, that elevated cholesterol is associated with the onset of CVD, therefore effective means to manage circulating cholesterol are key to reducing the risk of developing CVD.

1.2 Cholesterol Metabolism

Cholesterol is a 27-carbon molecule essential to the maintenance of cellular structure and function. It is a precursor for the synthesis of steroid hormones [21], oxysterols and bile acids [22], which are key components in cell signaling, sexual development and the regulation of lipid status [23]. The classic paradigm for the homeostatic regulation of the body's cholesterol pool is influenced by 6 key factors 1: endogenous cholesterol synthesis [24], 2: dietary cholesterol uptake [25] 3: biliary cholesterol excretion [26]4: intestinal cholesterol reabsorption and 5: faecal neutral sterol loss and 6: transintestinal cholesterol excretion (TICE) whereby cholesterol excretion bypasses the biliary route and is transported from the blood, directly to the intestinal lumen [8]. The scope of the current study has addressed factors 1 through 4, and briefly addresses factor 5. Adequate regulation of plasma and cellular cholesterol is imperative to maintaining overall homeostasis, due to the wide involvement of cholesterol and its associated intermediates to biological function. It is therefore not surprising that cholesterol synthesis, transport and excretion is carefully regulated at both transcriptional and post-transcriptional levels.

1.2.1 Key steps in endogenous cholesterol synthesis

The endogenous cholesterol synthesis pathway is a multi-step process (i.e. the mevalonate pathways), requiring sequential conversion of cholesterol pre-cursor molecules to its final form. The process begins with acetyl coenzyme A (acetyl-CoA), which is condensed with acetoacetyl CoA to form 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA), catalysed by HMG CoA synthase [27]. Analysis of endogenous cholesterol synthesis by mass isotopomer distribution analysis (MIDA) [28] utilises this step for incorporation of heavy labelled acetate into the cholesterol synthesis pathway, resulting in the formation of heavy labelled cholesterol.

Numerous studies have utilised this method [29, 30], and similar concepts [31] to determine the rate of endogenous cholesterol synthesis.

HMGCoA is then reduced to mevalonate, by HMGCoA reductase, which is well known as the rate limiting step of cholesterol biosynthesis. Early studies by Siperstein et al. [24] were the first to elucidate a negative feedback response in cholesterol synthesis, in response to cholesterol feeding. Since then, the cholesterol synthetic pathway has been further explored, confirming negative feedback regulation of cholesterol synthesis, facilitated by (Sterol Regulatory Binding Protein – 2) SREBP-2 in response to elevated levels of circulating cholesterol [32]. The current treatment for hypercholesterolaemia, statins, competitively inhibit HMGCoA reductase, thereby slowing the endogenous synthesis pathway at the rate limiting step [30] and decreasing circulating cholesterol concentrations.

Mevalonate kinase (MK), the second essential enzyme of the cholesterol synthesis pathway, facilitates the phosphorylation of mevalonate to 5-phosphomevalonate [33]. Phosphomevalonate kinase (PMK) catalyses the following step, facilitating the conversion of the phosphomevalonate to 5-pyrophospho-mevalonate and ADP in a reversible reaction. The formation of mevalonate is the first committed step of the cholesterol biosynthesis pathway, thereby acting as a target for potential treatment. Modulation of the mevalonate pathway is the current target for therapeutic approaches to cholesterol management and in some instances, cancer management [34], ultimately lowering circulating cholesterol concentration by reducing endogenous synthesis.

1.2.2 Exogenous cholesterol pathway

Alongside the endogenous pathway, ~30% of the body's total cholesterol is also derived from the exogenous pathway [35]. Exogenously derived cholesterol is an independent influencer of the rate of endogenous cholesterol synthesis, where increases in dietary cholesterol have been observed to modestly suppress endogenous cholesterol synthesis [36]. The exogenous cholesterol pathway utilises cholesterol derived from the diet and the bile, facilitating and regulating its absorption and incorporation into the circulation via the distal intestine, and transport back to the liver.

Absorption of dietary cholesterol begins in the intestinal lumen, where cholesterol esters derived from the diet are hydrolysed to free fatty acids (FFA's) and free cholesterol [35].
Uptake of cholesterol into the enterocyte occurs, facilitated by diffusion and protein mediated transport mechanisms, primarily for monoacylglycerols and FFA's. Uptake of dietary cholesterol into the enterocyte is mediated by the Niemann-Pick C1-like 1 (NPC1L1) protein, a transmembrane protein which harbours the cholesterol binding site [37]. In the enterocyte, esterification of free cholesterol is mediated by Acyl-CoA:cholesterol acyltransferase (ACAT) and subsequently packaged into Apo-B containing chylomicrons. These nascent chylomicrons are then sent to the golgi for further processing, then secreted via transporters located on the basolateral membrane for incorporation into the circulation. Additional transporters facilitate the generation of nascent HDL from Apo-A1 and exogenously derived cholesterol, namely ATP-binding cassette transporter A1 (ABCA1) [38].

1.3 Cholesterol Excretion - Bile formation and secretion

Bile formation and excretion is a key component of normal liver function, serving a number of important roles, including the excretion of exogenous and endogenous molecules. Cholesterol, being both exogenously acquired through the diet and endogenously produced, represents one of these metabolites, and is regulated by the liver.

1.3.1 An introduction to hepatic structure and function

The anatomical structure of the hepatocyte is a key determinant of biliary secretion, where different areas of the cell surface serve differing roles in the intracellular and extracellular transport of solutes. The cell surface of the hepatocytes consists of the basolateral (sinusoidal) membrane, which is the point of interaction between the blood (sinusoids) and the hepatocyte and the apical excretory domain, which forms the canalicular lumen when sealed with tight junctions to the apical surface of adjacent hepatocytes. The hepatocyte acts as the only barrier separating the blood and the bile and is heavily dependent on the function of the tight junctions, which seal the canalicular lumen, and prevents the diffusion of large solutes into the bile.

The canalicular membrane has 2 key functions: 1. Facilitate the transport and host biliary transporters that regulate secretion: 2. Withstand high concentrations of bile acids, which act as powerful detergents, capable of solubilize normal membranes. The components, such as phospholipids, within the canalicular membrane therefore play a key role in these functions. Movement of solutes across the apical membrane is dependent on the polar nature of the hepatocyte, where the positive region nears the apical membrane, basolateral domain of the cell is negatively charged. This characteristic of the hepatocyte facilitates the movement of small charged particles such as Ca^{2+} and sodium, to the canalicular lumen.

1.3.2 Hepatic cholesterol uptake

The hepatic uptake of low-density lipoproteins (LDL), intermediate density lipoproteins (IDL) and very low-density lipoproteins (VLDL) cholesterol is mediated primarily by the low-density lipoprotein receptor (LDLr). LDLr is expressed on the outer membrane of the cell in coated pits, where, upon interaction with apo-B100 [39], experience endocytotic internalisation, where endosomes are subsequently hydrolysed by lysosomes. Lipids are released into the cytoplasm, and endocytotic vesicles are then recycled back to the membrane. LDLr mediated internalisation is a key factor in the overall regulation of circulating cholesterol concentrations. Internalisation of LDL triggers several responses, including 1: reduced expression of HMG-COA reductase, to suppress endogenous cholesterol synthesis, 2: negative feedback suppression of LDLr synthesis via SREBP. The importance of the LDLr is demonstrated upon impaired function or expression of this receptor, which results in significantly elevated serum LDL, contributing to an accelerated onset of atherosclerosis [40]. An example of this is familial hypercholesterolaemia, where mutations in LDLr results in elevated LDL cholesterol [41].

1.4 Transporters of biliary lipid secretion

1.4.1 Biliary cholesterol transporters

Biliary secretion of cholesterol is the primary route for hepatic excretion and is mediated by active transport proteins described below.

Scavenger Receptor B type 1 (SR-BI)

Scavenger Receptor B type 1 (SR-BI) is a 82kDa transmembrane protein localised to the steroidogenic tissue and in the liver [38, 42] where it mediates selective uptake of cholesterol esters from HDL [43] , and mediates cholesterol efflux into the bile [42]. It is expressed on both the apical and basolateral membrane of the hepatocyte and is the primary pathway for the uptake of HDL derived cholesterol from the sinusoidal blood [44]. Uptake of cholesterol associated with HDL (HDL-C) occurs without the concomitant catabolism of the lipoprotein itself. Since HDL-C is the major contributor to biliary cholesterol, the localisation of SR-BI to apical membrane, would suggest a potential role in biliary secretion alongside other more commonly accepted mechanisms of biliary cholesterol excretion [44].

Interaction of SR-BI and HDL results in the reduction of circulating HDL concentrations, ultimately facilitating the removal of excess cholesterol from the periphery through HDL mediated transport [42]. The cellular expression of SR-BI is regulated at both the transcriptional and post-transcriptional levels [38]. Increases in biliary cholesterol excretion, and decreased plasma secretion, seen in overexpression of SR-BI (SR-BI-tg) [45] supports its role in the hepatic uptake of HDL cholesterol. Knockout and inhibition studies in mice have also observed a notable increase in circulating HDL cholesterol further confirming its definitive role in HDL metabolism [46]. It is commonly understood that HDL contributes to the protection against CVD and atherosclerosis, due to the ability of HDL to facilitate the removal of cholesterol from the peripheral tissue, however, paradoxical evidence suggests that the

overexpression of SR-BI, protects against the development of atherosclerosis by reducing plasma HDL [46, 47].

ATP-Dependent Binding Cassette Type G member 5/8 (ABCG5/8)

ABCG5/8 is a heterodimer transporter pair, responsible for the transport of cholesterol from the hepatocyte to the canalicular lumen. It is localised to the apical membrane of the hepatocyte but is also expressed in the intestine. In ABCG5 and/or ABCG8 knockout mice, Yu et al. [48] reported that biliary cholesterol content reduced by approximately 75%, suggesting that these transporters are, quantitatively, the major transporters of cholesterol into the bile. In turn, overexpression of ABCG5/8 results in the increase in biliary cholesterol [39, 49], which has also been explicitly associated with decreased risk of atherosclerosis [39]. ABCG5/8 is responsible for the active transport of cholesterol into the canalicular membrane

Niemann Pick c1-Like Protein (NPC1L1)

Niemann Pick c1-like Protein (NPC1L1) is a regulator of cholesterol absorption in the intestine but is also heavily expressed in human liver [37] . In the hepatocyte, NPC1L1 is located on the canalicular membrane, suggesting a role in biliary transport [37]. NPC1L1 facilitates the export of cholesterol from the intestinal lumen, and plays a similar role across the canalicular membrane, facilitating the reuptake of newly secreted cholesterol across the canalicular membrane. In situations of overexpression, ie. NPC1L1 transgenic mice with overexpression of the transgene, a significant decrease in biliary cholesterol concentration was observed, while biliary phospholipid and bile acids remained unaffected. This would suggest an increase in cholesterol specific processes, particularly flux from the canalicular lumen, back into the hepatocyte. Ezetimibe, a drug commonly used for the management of hypercholesterolemia, targets NPC1L1, inhibiting reuptake thereby increasing biliary excretion [43, 50]. A

randomisation study by Ference et al. [34] revealed NPC1L1 polymorphisms by Ezetimibe, result in proportionate reduction in circulating LDL, highlighting a potential dose dependent effect. This information therefore highlights Ezetimibe as a viable treatment option for hypercholesterolaemia, where it is currently used in conjunction with statins [5, 51]

1.4.2 Biliary Phospholipid Transporters

Phospholipids are key molecules involved in biliary lipid secretion, alongside cholesterol and bile acids. The phospholipid content of the canalicular membrane is key to its maintenance and integrity, contributing to membrane resistance to the detergent-like activity of bile acids.

ATP-Dependent Binding Cassette Type B member 4 (ABCB4)

ATP-Dependent Binding Cassette Type B member 4 (ABCB4), is otherwise known as multidrug resistance P-glycoprotein 2 (MDR2; Mdr2 in rodents) [43]. ABCB4 is an ATP-dependent phospholipid flippase protein, involved in the translocation of phospholipids, from the inner leaflet of the canalicular membrane, to the outer leaflet [52]. It is believed that this flippase activity, which has a high efficacy for phosphatidylcholine (PC) transport, exposes the phospholipid to the canalicular bile flow, making it readily available for extraction directly from the canalicular membrane. Disruption of the Mdr2 gene in ABCB4 knockout mice, results in the complete absence of phospholipid secretion into the bile [53]. Despite its well-documented phospholipid specific activity, this study showed that absence of ABCB4 activity resulted in virtually no biliary cholesterol excretion, regardless of bile salt concentrations in the bile [52]. It is hypothesised that the increase PC on the outer leaflet of the canalicular membrane results in the formation of “outpouches” which may encourage the extraction of lipids from the membrane.

ATPase Phospholipid Transporting 8B1 (ATP8B1)

Contrasting the activity of ABCB4, ATPase Phospholipid Transporting 8B1 (ATP8B1) is a p-type ATPase that flops phospholipid, particularly phosphatidylserine (PS) form the outer leaflet of the canalicular membrane, to the inner leaflet [43]. This results in a decrease in PS content of the outer leaflet, and a subsequent increase in sphingomyelin content. Disruption of this transporter results in enhanced biliary phospholipid excretion, and paired with this, increased cholesterol excretion [54]. It is believed that this is due to the decreased rigidity of the outer leaflet, allowing for easier lipid extraction, and subsequent formation of mixed micelles. Mutations in the ATP8B1 gene in humans are commonly associated with hereditary cholestasis, particularly progressive familial intrahepatic cholestasis (PFIC) [54] characterised by low biliary bile salt concentrations, elevated serum bilirubin concentration and normal cholesterol levels. In mouse models, deficiency of the flippase results in impaired bile salt transport [55]. Although the mechanism by which ATP8B1 deficiency results in cholestasis isn't entirely clear, it has been hypothesised that the asymmetry between leaflets of the canalicular membrane is not maintained, thereby impairing hepatobiliary bile salt transport, on the basis of coupled excretion between bile salts and biliary lipids (cholesterol and phospholipids) [56]. This coupling is further supported by the closely related activity of ATP8B1 with ABCB11, a transporter primarily responsible for bile acid secretion.

1.4.3 Biliary bile acid transporters

ABCB11

ATP-Dependent Binding Cassette Type B member 11 (ABCB11) is more commonly known as the bile salt export pump (BSEP) and is the primary mediator of biliary output of bile salts

[43]. Studies exploring overexpression of ABCB11 showed a substantial increase in biliary bile acid secretion, where expression of other biliary transporters remained unchanged [57]. Despite this, the overexpression of ABCB11 results in the promotion of hypercholesterolaemia and associated obesity in mouse models, which contrasts the commonly accepted phenomenon of coupled secretion of cholesterol and bile acids. Other studies however, showed that ABCB11 is not the only route of biliary bile acid secretion, where ABCB11 deficient animals showed upregulation of ABCB1a/b and residual secretion, inferring a compensatory mechanism [57, 58]. ABCB11 plays an important role in the regulation of bile acid secretion, where activation of Farnesoid X Receptor (FXR) results in the downregulation of bile acid synthesis, and the increase in bile acid secretion, by activation of ABCB11.

Figure 1.3 *Overview of canalicular transporters/proteins involved in biliary secretion of bile acids, cholesterol and phospholipids [43].*

1.5 Cholesterol and atherogenesis

Hypercholesterolaemia is a primary risk factor for CVD, mainly due to its direct contribution to the onset of atherosclerosis [59]. In hypercholesterolemic conditions, the risk of atherosclerosis increases, as more cholesterol is readily available for deposition into plaques on the inner walls of the vasculature [18]. The sequelae associated with atherosclerosis include embolism and blockage of medium sized arteries, leading to myocardial infarction and stroke [60]. A key contributor to the development of atherosclerosis, is the oxidative modification of cholesterol.

Figure 1.2 *Atherogenesis- Production of. ROS, promotes the oxidation of LDL, where it is transported to the cell for uptake by the macrophage. OxLDL promotes the formation of macrophage foam cells, which form the necrotic core of atherosclerotic lesions.[29]*

1.5.1 Oxidative stress - a key contributor to atherosclerosis

The generation of excessive free radicals contributes to the oxidation of proteins and lipids, as well as damage to the endothelium, therefore resulting in oxidative damage, inflammation and loss of cellular function [61]. Oxidation of macromolecules such as lipids, proteins [62] and nucleic acids may contribute to the pathogenesis of many diseases. The *Oxidative Modification Hypothesis*, originally proposed by Steinberg [63] in 1989, describes the involvement of oxidised LDL (oxLDL) in the development of atherosclerosis as it activates endothelial cells, macrophages, and smooth muscle cells [64], and with the interaction of scavenger receptors, produce foam cells. OxLDL induces the apoptosis of vascular smooth muscle cells (VSMCs), with increased cellular cholesterol, necrosis and remodeling causing rupture of the atherosclerotic plaque, resulting in more serious complications. Liu et al. [28] noted that increased oxLDL resulted in increased proliferation and migration of VSMCs to the site of injury, thus contributing to the size of the atherosclerotic lesion (Figure 1.2).

1.5.2 Current treatments for hypercholesterolaemia - Statins

Statins are currently used in individuals at increased risk of cardiovascular disease. Statin administration elicits salutary effects on endothelial function, inflammation [65, 66] and modulates lipid profile [65]. When statins bind to HMG-CoA reductase, they inhibit the binding of natural substrate HMG-CoA, thereby preventing the conversion of HMG-CoA to mevalonate and thus inhibiting cholesterol synthesis [67]. Additional benefits of statin use include favourable (increasing) bioavailability of nitric oxide (NO) [68], and subsequent improvement of endothelial function [69]. Studies investigating the efficacy of different types of statins [70-73] have also outlined their effects on lipid profile in various human populations. For example, statins reduce LDL levels by 20-55% (35) depending the type of statin

administered. It is commonly accepted that statin treatments increase circulating HDL cholesterol concentrations, as demonstrated in numerous rodent and human studies. Reductions in HDL and TG may also occur at higher doses, by 6-12% and 10-29% respectively (35,36).

Recent studies have explored the mechanism by which statins reduce circulating cholesterol, beyond inhibition of the mevalonate pathway. A study by Schonewille [30] explored the effects of statin therapy in mice, paradoxically noting an overall increase in cholesterol synthesis, as well as organ specific cholesterol synthesis. This resulted in subsequent increase in faecal cholesterol excretion, which was proposed as the key mechanism responsible for cholesterol reduction. Similar to the current study, Schonewille and colleagues utilised MIDA [74] as a means for assessing de novo cholesterol biosynthesis, by assessing the concentration of heavy labelled cholesterol [26] as a ratio to non-labelled cholesterol. This paradoxical observation in endogenous synthesis resulted, supposedly, as a response to an accumulation of HMG-CoA and an upregulation of SREBP-2, ultimately increasing the amount of cholesterol endogenously synthesised. Furthermore, it has been previously reported that faecal excretion of cholesterol had increased [72] during statin therapy, which also paralleled the findings in Schonewille's study. Similar mechanisms occur with the use of other cholesterol lowering drugs, namely Ezetimibe [75, 76] and bile acid sequestrants [29].

1.6 Key Determinants of bilirubin metabolism

Historically, elevations in circulating bilirubin were associated with the condition of neonatal jaundice, which was first described in detail in the early 18th century [77]. Since then, the progression of knowledge surrounding bilirubin and its effect on the body has resulted in its use as a diagnostic tool for assessing liver dys/function and associated mortality [78]. During the last four decades however, knowledge of the *in vivo* importance of bilirubin has proved

involvement in neuronal function [79], and more recently, and prevention of CVD related morbidities, atherosclerosis and hypercholesterolaemia [80]. Observational studies have also noted an inverse relationship between serum bilirubin concentrations and circulating cholesterol concentrations [81-85]. The following sections describe the formation and regulation of circulating bilirubin concentrations, which are critical to understanding the potential of this molecule to modulate its potential beneficial effects.

1.6.1 Biosynthesis of bilirubin - Haem catabolism

The synthesis of bilirubin results from the degradation of the haem, commonly obtained from haemoglobin [86], amongst other haem-containing proteins. Approximately 80% of bilirubin originates from the degradation of erythrocyte derived haem, and the remaining 20% from insufficient erythropoiesis and degradation of other haem containing proteins [87]. In areas of red blood cell turnover, such as the bone marrow, spleen and in the liver, phagocytosis of erythrocytes by localised macrophages results in the structural breakdown of haemoglobin to two main components; haem, and globin. Haem is then metabolised by haem oxygenase (HO) [86], and required oxygen and reducing equivalents for catabolism. There are three known HO isoforms including HO-1, HO-2 and HO-3, with HO-1 being the key contributor to the formation of bilirubin, and is expressed in states of metabolic and oxidative stress [88]. Haem oxygenase mediated haem degradation results in the production biliverdin IXα by cleavage of haem ring at the α-methene carbon bridge, simultaneously yielding other byproducts, carbon monoxide and iron [86]. Biliverdin IXα is rapidly reduced by biliverdin reductase (BVR), which reduces γ-meso bridge of biliverdin to bilirubin (unconjugated bilirubin) [89, 90]. In instances of HO-1 inhibition, production and therefore the circulating concentration of unconjugated bilirubin (UCB) decreases, indicating that this enzyme the rate limiting step in the degradation of haem and in the synthesis of bilirubin [89, 90]. HO-1 activity is inhibited by biliverdin mediated negative feedback, where biliverdin inhibits the oxidation of haem [90].

Regulation of circulating biliverdin concentrations by BVR, and the regulation of HO-1 by biliverdin suggests coupled catalytic activity, resulting optimal clearance of free haem, which can be cytotoxic [3].

Figure 1.4. *Diagrammatic representation of bilirubin synthesis from Haem. Haem is degraded by haem-oxygenase at the α-methene carbon bridge forming biliverdin IXα, then reduced to bilirubin by biliverdin reductase [91].*

1.6.2 Bilirubin transport

Unconjugated bilirubin has a very low aqueous solubility, and is lipophilic at physiological pH, due to the number of hydrophobic groups, and the hydrogen bonding of its carboxylic acid groups. Therefore, in the circulation, UCB is bound by at least one albumin binding site. On this basis, UCB measured in the plasma is most commonly bound to albumin, with free bilirubin only detected in nanomolar amounts. The bilirubin-albumin binding complex has additional benefits, preventing the oxidative damage to albumin and the vasculature [92, 93].

1.6.3 Bilirubin conjugation

Following uptake of bilirubin into the hepatocyte, bilirubin is readily conjugated with glucuronic acid, to either bilirubin monoglucuronide or diglucuronide in the endoplasmic reticulum [93]. Glucuronidation of bilirubin increases the compound's water solubility allowing for its incorporation into the bile for excretion. The key enzyme responsible for the conjugation of bilirubin is (UDP Glucuronosyltransferase Family 1 Member A1) UGT1A1[87, 94, 95]. Impairment of UGT1A1 activity causes reduced conjugation, and as a result, an accumulation of unconjugated bilirubin in the circulation and systemic tissues. Impairment or complete inhibition of UGT1A1 activity is the genetic basis describing Gilbert's and Crigler Najjar's syndrome, respectively [96, 97].

1.6.4 Bilirubin excretion

Upon interaction with the basolateral membrane of the hepatocyte, bilirubin is released from albumin and enters the hepatocyte by means that have not been fully elucidated [98, 99]. The lipophilic nature of bilirubin would suggest passive diffusion across the membrane, however, the specificity of transport into the hepatocyte does not agree with this conclusion [97]. Hepatic uptake of albumin bound bilirubin (unconjugated) in humans is facilitated by organic anion-transporting polypeptide 2 (OATP2 or OATP1B1), and OATP8 (or OATP1B3) to a lesser degree [98]. Studies have noted that OATP2 has a higher affinity for bilirubin uptake than OATP8, differentiated by their capacity to remove albumin, and thus transport free bilirubin [98]. The conjugation process results in a water-soluble product, either as bilirubin monoglucuronide or diglucuronide [100]. Secretion of bilirubin across the cannalicular

membrane is mediated by multidrug resistant protein 1 (MRP2), an ATP-dependent, unidirectional efflux pump that is expressed predominantly in the hepatocyte, and localised to the apical domain [98, 101]. Inhibition experiments of human MRP2 and the equivalent rat homolog (mrp2), have resulted in conjugated hyperbilirubinemia [97, 102], mimicking the phenotype seen in those with Dubin-Johnson Syndrome and Groningen Yellow (GY) rats respectively.

Bilirubin excretion via the kidney serves as an alternative pathway for the removal of bilirubin, particularly in instances of impaired hepatic bilirubin secretion [103]. Unlike UCB, conjugated bilirubin is not bound to albumin in the circulation and is readily excreted via the urine. UCB on the other hand is transported to the proximal tubular cells by OATPs, where it is conjugated by UGT1A1 and excreted in the urine [103].

The intestine plays a notable role in the overall regulation of plasma bilirubin, serving as an alternate excretory pathway, especially in instances of unconjugated hyperbilirubinemia [8]. Reabsorption of bilirubin for incorporation back into the enterohepatic circulation occurs via passive or paracellular diffusion [104, 105] affecting circulating bilirubin concentrations.

1.6.5 Disorders in bilirubin conjugation

Gilbert's syndrome

Gilbert's Syndrome (GS) is described as a benign, yet mild elevation in circulating unconjugated bilirubin [106, 107]. This condition is associated with the presence of an autosomal recessive genotype affecting the UGT1A1 gene promoter, occurring in homozygous or compound heterozygous individuals [96, 108]. Mutations in UGT1A1 include a dinucleotide polymorphism in the TATA box promoter region of the UGT1A1 gene. The genetic

polymorphism, known as UGT1A1*28 [107], is a TA insertion in the TATAA box of the UGT1A1 promoter, and causes an ~50% decrease in UGT1A1 activity [96, 108]. This effect is due to a lessened affinity of the TATAA-binding protein to the TATAA box, therefore decreasing expression of the gene which encodes for UGT1A1 [96, 97]. In addition to impaired UGT1A1 activity, Gilbert's syndrome is also associated with impaired hepatic uptake of bilirubin, mediated primarily by OATP2 [101]. Intermittent jaundice is currently the only clinically recognised symptom however, more recent studies have elucidated additional characteristics associated with UGT1A1 impairment, in humans and murine models, including protection against CVD.

The prevalence of Gilbert's syndrome mutations seems to vary amongst ancestral groups with studies suggesting evolutionary adaptation of circulating bilirubin concentration and therefore highlighting the potentially benefits of mild hyperbilirubinemia [107]. Populations with Caucasian ancestry have a ~2-10% prevalence of GS, whereas populations of middle eastern descent have prevalence percentages of approximately 20% [107]. Genetic studies on various ancestral backgrounds have found greater discrepancies between them, with African populations possessing the highest percentage (23%) of UGT1A1*28 mutations, whereas Asian populations show only 3% prevalence of the UGT1A1*28 mutation [108]. The diagnostic definition of GS varies amongst different organisations, therefore creating uncertainty regarding the prevalence of this condition [107]. Circulating total bilirubin concentrations of >17uM to >22uM with otherwise normal liver function, is a general diagnostic description of Gilbert's syndrome in humans [8], describing a mild elevation in circulating bilirubin when compared to the general population (~10 μM) [96].

Crigler Najjar- Syndrome

A more severe disorder of impaired bilirubin glucuronidation is characterised by Crigler Najjar (CN) Syndrome. This disorder generally occurs as one of 2 variations CN-1 and CN-2, describing complete and partial loss of UGT1A1 activity, respectively [109]. CN is the most lethal form of hereditary hyperbilirubinemia, characterised by severely elevated levels of serum bilirubin to levels reaching 100-855 μM [97], which represents a 10-80-fold increase compared to normal circulating bilirubin levels [110].

If left untreated, individuals with Crigler Najjar Syndrome are at risk of neuronal toxicity, caused by the transfer of free unconjugated bilirubin from the extracellular space of the brain, to the intracellular space [79, 97]. An accumulation of free bilirubin in the brain, potentially causes irreversible brain damage and associated complications such a sensory hindrance [79]. Individuals with CN must maintain control of their bilirubin levels, beginning with aggressive phototherapy at the early stages of development, however at adulthood, the only definitive treatment available is a liver transplant [111, 112]. Failure to treat severe hyperbilirubinemia, could result in severe brain damage, kernicterus or death.

1.6.6 Disorders in biliary excretion

Dubin Johnson Syndrome

Unlike Gilbert's and Crigler Najjar syndrome, Dubin Johnson syndrome is described as a genetic perturbation of biliary transport of bilirubin glucuronides and other organic anions, excluding bile acids. A mutation in the ABCC2 gene, which encodes for the transport protein MRP2, results in the insufficient export of conjugated bilirubin form the hepatocyte to the bile, resulting in the concurrent conjugated and unconjugated hyperbilirubinemia (~50-100uM) [101]. Similarly, to Gilbert's syndrome, the congenital mutation is autosomal recessive, but does incur additional characteristics, including a black coloured liver [110, 113]. Rat models,

including the Groningen Yellow Rat (TR-), Esai-hyperbilirubinemic (EHBR) rat), that also possess an autosomal recessive defect in transporter proteins (mrp2), have been compared with human Dubin Johnson syndrome [101] and have been used to improve our understanding of the molecular basis and implications of this condition. Fortunately, Dubin Johnson syndrome is considered benign, due to its relatively low/moderate degree of unconjugated hyperbilirubinemia [110, 113].

1.7 Hyperbilirubinaemia and lipid metabolism

The historical assumption that bilirubin is a simply a byproduct of haem catabolism has evolved over the past decades, displaying favourable involvement in endothelial function, atherogenesis and a decrease in all-cause mortality [10, 114]. Since the association of bilirubin and cardiovascular disease was first proposed by Schwertner in 1994, [115] numerous studies have identified associations between hyperbilirubinaemia with protection against cardiovascular disease, [80, 115-117] Despite these findings, the key mechanisms responsible for bilirubin potential protective effects have not been explicitly elucidated.

Bilirubin is strongly implicated in protection against atherosclerosis, where factors such as inflammation, lipid status and antioxidant capacity all contribute to reduced prevalence and delayed onset in hyperbilirubinemic subjects [8]. Beneficial effects on vascular structure are associated with mild elevation in serum bilirubin, indicated by reduced intima media thickness in the aorta [118] and the coronary artery [115] in hyperbilirubinaemic individuals. Carotid intima media thickness is strongly correlated with an increased risk of CVD [119], suggesting that reduction of intima media thickness (IMT) by bilirubin, could decrease CVD and death

[120]. The antioxidant capacity of bilirubin is a commonly assumed mechanism by which bilirubin exerts its cardiovascular protective effects [121, 122] These antioxidant effects could also prevent or attenuate hypertension, by improving NO bioavailability, likely providing protection from related cardiovascular conditions including cardiac failure, arterial dissection, aneurysm and haemorrhagic stroke [123].

Aside from bilirubin's antioxidant capacity alternative mechanisms contributing to protection from atherosclerosis, likely exist. For example, circulating cholesterol and lipid concentrations are considered primary risk factors for coronary artery disease. Interestingly, several human trials have demonstrated significant reductions in total and LDL concentrations, in addition to reductions in body mass [8]. Human studies also report negative associations between circulating bilirubin and apo-b containing lipoproteins, namely remnant chylomicrons [124], LDL, and VLDL. These findings suggest an alternative pathway for atheroprotection in Gilbert's individuals, as reductions in circulating plasma cholesterol decreases the prevalence of atherosclerotic lesions [125]. Many studies have previously outlined the favourable lipid profiles in hyperbilirubinemic human and murine models [8], however the cause of the reductions commonly seen in plasma lipid concentrations of hyperbilirubinemia models remains elusive.

Studies reporting the mortality and longevity of individuals with mild hyperbilirubinemia demonstrate an ~50% reduction in all-cause mortality in GS, compared to controls [10]. Longevity in GS individuals, has recently been explored in elderly populations, with cross sectional data indicating a U-shaped relationship between age of death and bilirubin concentrations in males. Interestingly, the opposite effect was seen in females, suggesting mild elevations in circulating bilirubin, contributes to greater longevity in males only [125].

Biliary cholesterol secretion is also altered in condition of increased biliary bilirubin secretion. The well-known phenomenon of 'coupled' biliary excretion, means that biliary cholesterol secretion is dependent on the secretion of phospholipids, bile acids and organic anions, including conjugated bilirubin [126]. Uncoupling of biliary lipid secretion reduces biliary cholesterol section, whilst bile salt concentrations remain relatively unchanged [126, 127]. Coupled biliary secretion between lipid (cholesterol + phospholipid) and bile salts is perturbed, by interaction between bilirubin and bile salts. This disrupts the detergent-like properties of bile salts, thereby reducing their ability to extract lipids from the canalicular membrane, by facilitating micelle formation. Apstein et al [128] was the first to explore the effect of bilirubin in biliary lipid excretion in Gunn rats, providing convincing evidence that the lipid lowering effect of bilirubin (bilirubin ditaurate, BDT) occurred within the biliary lumen. Verkade et al, then followed, further progressing to models of conjugated hyperbilirubinaemia [129, 130]. These studies showed that infusion of water soluble BDT was strongly and negatively correlated with the lipid:bile acid ratio. These data suggested that infusion of BDT caused a reduction in absolute biliary lipid secretion in a dose dependent manner [128]. These findings were supported by Kajihara et al. [131], who reported similar decreases in biliary cholesterol, without inducing any change in bile acid output in response to BDT infusion.

Bilirubin and atherosclerosis

Recent data suggests that mildly elevated concentrations of circulating bilirubin are associated with protection against various oxidative stress-mediated diseases, particularly in the development of atherosclerosis [132-134].

1.7.1 Antioxidant effect of bilirubin

One of the key cardiovascular protective effects of bilirubin, is believed to reside within its powerful antioxidant and inflammatory potential [132]. Bilirubin is a potent endogenous antioxidant, protecting against the formation and effects of free radical production [135]. Biomarkers of oxidative stress include oxidised low density lipoproteins (oxLDL), which have been observed to have a negative correlation with total bilirubin in the circulation, in both human and murine models [121, 136]. The antioxidant potential of bilirubin was first alluded to by Stocker et al. [92] who observed that micromolar concentrations of bilirubin prevented the oxidation of albumin and its bound fatty acids. A key study by Wu et al [137] highlighted the high potency of bilirubin as an antioxidant, demonstrating that bilirubin, at physiological concentrations, was approximately 20 times more effective in preventing LDL oxidation than a vitamin E analogue, Trolox. These studies, however, were tested *in vitro* and therefore, had limited relevance to the *in vivo* condition. The antioxidant effects of bilirubin have more recently been assessed in several tissues, including the blood [121, 136] and endothelia [138] and intact animals [139]. Ziberna et.al [138] further confirmed role of the vascular endothelium as the "transducer" for bilirubin's antioxidant effects *in vitro*, thereby contributing to the protective effects of bilirubin on endothelial function and cardiovascular disease.

1.7.2 Bilirubin and endothelial function

Bilirubin may hinder the process of atherosclerotic development, by maintaining and protecting the integrity of the endothelial wall. Endothelial damage is one of the key initiating factors for the development of atherosclerotic lesions, therefore attenuation of endothelial dysfunction and damage could delay and slow atherosclerosis. Previous studies regarding bilirubin mediated protection of endothelial function include alteration in flow mediated dilation [140] and prevention of endothelial sloughing [141], which appear to be mediated by redox dependent

mechanisms. Currently there is only one animal study that explicitly outlines the protective effects of bilirubin, against atherosclerosis [142]. Wang et al. [143] also demonstrated a negative correlation between bilirubin and hypertension, where structure and integrity of the endothelial wall was associated with the heightened antioxidant capacity.

1.7.3 Bilirubin and lipid status

It is believed that the favourable lipid status seen in individuals with Gilbert's syndrome, is mediated by increased bilirubin affecting regulatory mechanisms of cholesterol metabolism [8]. Decreased circulating cholesterol concentrations are commonly observed in GS [81, 82, 136, 144] including reductions in non-HDL cholesterol [81, 82, 136, 144]. However, Vitek et al. [84] also reported increased total cholesterol and LDL in GS. However, it should be noted that in this study group, the GS and control groups possessed different gender distributions (male = 35, female 15) and control (male = 33, female = 5) which may have influenced the results. Bilirubin accumulates in macrophages and foam cells, and decreases cholesterol efflux from macrophages, by ABCA1, which is a transporter responsible for the export of HDL cholesterol from the periphery (and intestine) to the circulation [145]. Ultimately this could decrease the cholesterol transported to the circulation, subsequently lowering total cholesterol concentrations.

These findings suggest an alternative pathway for atheroprotection in GS individuals, beyond bilirubin's accepted antioxidant effects, with mild hypocholesterolaemia potentially decreasing the prevalence of overt atherosclerosis. Given that it is difficult to assess mechanistic causes of altered cholesterol metabolism in human studies, this had led investigators to use hyperbilirubinaemic animal models (including the hyperbilirubinaemic Gunn rat) to better understand potential role of bilirubin in modification of lipid status.

Currently, few studies have explicitly investigated lipid status in Gunn rats. However, in those published studies, Gunn rats consistently show reductions in circulating LDL/non HDL cholesterol [81, 82]. Furthermore, reduction in total cholesterol were reported in all Gunn rat studies [81, 136, 144], with reductions in HDL reported by Wallner et.al [81] and Boon et.al [136]. Interestingly, Wallner et.al. [81] observed a more prominent reduction in circulating lipids in female Gunn rats, thereby suggesting the possible influence by sex specific hormones on lipid status. The current thesis aims to explore this difference further, noting the effect of unconjugated hyperbilirubinemia on cholesterol synthesis and hepatic biliary excretion, which could help to determine whether bilirubin affects cholesterol production, or excretion. These data could therefore, help to explain why circulating cholesterol species are altered in conditions of benign hyperbilirubinaemia and guide future mechanistic investigations.

2.0 Aims Objectives and Hypotheses

2.1 Aims

Aim 1: To identify potential differences in endogenous hepatic cholesterol biosynthesis, between hyperbilirubinaemic homozygote Gunn and normobilirubinaemic heterozygote Wistar (control) rats.

Aim 2: Investigate modulation of biliary cholesterol and lipid excretion in Gunn and control rats.

Aim 3: To report sex specific differences in endogenous cholesterol synthesis and biliary lipid excretion, in Gunn and control rats.

2.2 Objectives

The primary aim of this study was to explore differences in de novo cholesterol synthesis, in littermate Gunn and control animals. This was done by utilising the method outlined by Verkade [26], where heavy labelled acetate is incorporated into the cholesterol biosynthesis pathway, allowing endogenously produced cholesterol to be distinguished easily from exogenously sourced cholesterol. Factors such a faecal cholesterol content and biliary cholesterol excretion were also be considered to further clarify any modulation in cholesterol metabolism.

The second aim was to examine potential differences in cholesterol excretion between Gunn and control rats. Biliary and faecal excretion of cholesterol, phospholipids and bile acids were assessed using routine biochemical analyses. Analysis of the bile, and of bile flow allowed for calculation of cholesterol and lipid excretion, as described previously. Additional variables such as total and direct bilirubin were also analysed to validate the phenotype of Gunn rats.

Additional factors such as the hepatic cholesterol content were included to provide insight into any potential differences in cholesterol transport, in response to a deficiency in UGT1A1, or hyperbilirubinaemia. Analysis of these additional factors gave insight into the function of other influential pathways in cholesterol metabolism, as well as strengthen in analysis of endogenous cholesterol biosynthesis and assist in determining the cause of the altered lipid status [8].

The third aim of this study was to explore sex specific differences in cholesterol metabolism in Gunn and control rats. This aim was primarily achieved during the data analysis phase by analysing the above variables in male and female Gunn compared to control animals. Furthermore, analysis of pre-pubescent juvenile (~3 weeks old) animal serum was also used to supplement the findings seen in the adult study. Juvenile animals were investigated to assess whether differences observed in adult male and female animals, which may have been mediated by sex hormones, remained present in animals who were not sexually mature.

2.3 Hypotheses

Hypothesis 1:

Null: There is no significant difference in de novo cholesterol biosynthesis, when comparing Gunn and control animals.

Alternative: Gunn rats possess reduced do novo cholesterol synthesis, compared to control animals.

Hypothesis 2:

Null: There is no significant difference in biliary lipid, or bilirubin excretion, when comparing Gunn and control animals.

Alternative: Gunn rats possess increased biliary lipid excretion compared to control animals.

Hypothesis 3:

Null: There is no significant difference in de novo cholesterol biosynthesis, between male and female, Gunn and control groups

Alternative: Female Gunn Rats (compared to male) experience significant reductions in de novo cholesterol synthesis and biliary lipid excretion.

3.0 Methods

Before Terminal

Animals and Housing

Male and female rats of desired phenotypic distribution were obtained from Dr Bulmer's Gunn rat breeding colony (Breeding pair 3A; homozygous male, heterozygous (Wistar) female). Animals were housed in the G26 animal house facility at Griffith University. 36 age-matched (76 day old) animals (Control; male n=8, female n=9. Gunn; male n=9, female n=10) were housed and raised from four litters and entered protocol, with groups of 3 animals staggered one day apart. Animals that are 10+ weeks old will be referred to as 'adult' for the purpose of this thesis. Animals were housed in a 12hr light-dark cycle in constant temperature (20°C), and humidity (60%). Homozygous animals were phenotyped by jaundice at birth, ear tagged and at weaning, housed with the littermates of the same sex. Animals were fed a standard rodent diet (TEKLAD Standard Global 18% Protein Rodent Diet: Appendix 1) and fresh water *ad libitum.* Animal cages were changed regularly as per animal house maintenance and tissue boxes included in the cages to ensure well-being of the animals. All procedures were performed following appropriate training. All procedures complied with *Australian code for the care and use of animals for scientific purpose* (NHMRC) and Animal Ethics Approval (MSC/02/17/AEC) which was obtained from Griffith University prior to initiating the project

Experimental Protocol

At 10 weeks old, animals entered into the approved protocol. The protocol included 24 hour periods in metabolic cage at days -4, -1, 10, 14 and 18 of the 19 day protocol. Body weight of each animal was recorded every two days from day 0 to day 19. 400µL drinking water was administered via oral gavage (for the purpose of controlling for interventions applied in other studies) daily from day 0 to day 19, and sterile phosphate buffered saline (400µl) was

administered every 2 days via intraperitoneal injection for the purpose of controlling for interventions applied in other studies. Blood samples (approx. 1mL) were collected at day 1 and day 8 after fasting for a period of six hours, via tail bleed (see "Blood collection; tail bleed), and blood spots collected twice daily for the final 5 days of the study (see Blood collection; blood spot collection). During the final 5 days of the protocol acetate supplementation occurred. On the final day of the study, animals underwent surgery after anaesthesia (see Bile duct cannulation) and were euthanized via removal of the heart.

Metabolic Cages

Urinary and faecal excretion, food and water consumption were monitored using metabolic cages. Prior to the commencement of the study, animals were placed in metabolic cages (Day 1: 2 hours, Day 2: 5 Hours) to familiarise the animals with the environment. When entered into the protocol and on scheduled timepoints, animals were placed in the metabolic cages for a period of 24 hours. Whilst in metabolic cages, food (65-75g TEKLAD Standard Global 18% Protein Rodent Diet, 18% protein, 6.2% fat) and drinking water (<300g) were readily available for the animals to consume *ad libitum*. The weights (g) of the food and water were recorded before and after the 24-hour period, and the difference used to determine the consumption of food and water. Faeces was cleaned of food debris and weighed immediately then air dried at room temperature for 48 hours in a fume cabinet. The weight was recorded, and faeces stored at room temperature in an airtight container for later analysis. A small portion (~2mL) of urine was centrifuged at 3901 g, and 1mL stored at -80°C for later analysis.

Administration of C13 acetate

Drinking water was supplemented with [13 C]-acetate, a stable, non-radioactive heavy labelled isotope of acetate, a precursor for endogenous cholesterol and fatty acid synthesis. In the final

5 days of the study, water was supplemented with 2% (w/v; 2g/100mL) of [13 C]-acetate, and the amount consumed in the first 12 hours was recorded.

Blood Spot/Tail Bleed

Animals were fasted for 4-6 hours prior to blood collection. Animals were then anaesthetised with 2-5% isofluorane (IsoFlo) in 100% oxygen via inhalation (1-2 L/min). Pedal reflexes were tested to ensure the animals were not pain responsive. The tip of the tail (1-2mm) was removed with a sterilised scalpel blade and blood was drawn via gentle massage of the tail. The first few drops of blood were discarded before collecting 1mL of blood in a 1.5 mL Eppendorf. Blood was then left to stand for approx. 10 min at 4°C before centrifuging (2000g 10 minutes, 21°C). Serum was flash frozen in liquid nitrogen and stored at -80°C for later analysis.

Blood was drawn with tail bleed where blood spots (> 6 mm diameter) were taken on Whatmann no.1 Filter paper and air dried for later analysis (2 drops ~20µL). Blood spots were collected 0, 24, 36, 48, 60, 72, 84, 96, 108, 120 hrs via tail bleed (see Blood collection) after commencing [C^{13}]-acetate supplementation [26].

During Terminal

Bile duct Cannulation

On day 19 of the protocol, animals were fasted 6-7 hours prior to anaesthetisation. Rats were anaesthetised using a ketamine (50mg/kg) and xylazine (3mg/kg) mixture via intraperitoneal injection. Initial dosage was based on the weight of the animal (heterozygous 1.3µL/g, homozygous 1µL/g) and additional anaesthetic administered to achieve a state at which the animal was still breathing but did not demonstrate a pain response. Pedal reflexes were routinely checked prior to and during the procedure to ensure the animal had reached a surgical plane of anaesthesia. Body temperature was maintained using a heating pad, and rectal temperature monitored throughout the procedure. A midline laparotomy allowed access and

identification of the common bile duct, with the aid of a dissecting microscope. The bile duct was cannulated with 0.78 OD x 0.32 ID mm tubing (Microtube Extrusions, Australia) and secured in place with a drop of superglue and sutures. Once the bile duct was secured, the abdomen was closed and secured with sutures to maintain body temperature. The first 5 minutes of bile collected was discarded. Bile was then collected for 30 minutes in pre-weighed 1.5mL polypropylene tubes. 100µL of bile was then aliquoted into a 1.5mL pre-weighed Eppendorf tube and the weight recorded. Bile samples were immediately frozen in liquid nitrogen. Tissue collection occurred as per protocol (Appendix 2, Tissue collection protocol). Blood was collected from the inferior vena cava and immediately transferred to lithium heparin tubes and centrifuged (2000g, 10 minutes, 4°C), before the supernatant was extracted and flash frozen with liquid nitrogen. Blood collected as per: Blood sample preparation. Liver tissue was rinsed with cold Dulbecco's Phosphate-Buffered Saline (DPBS) (Sigma Aldridge, Australia) before being flash frozen in liquid nitrogen.

Juvenile Tissue Collection

Animal distribution consisted of Gunn (male n=10, female n=7; homozygous) and control (male=n=8, female n=5; heterozygous) animals. Animals that are 3-4 weeks old will be referred to as ‘juvenile’ for the purpose of this thesis. Animals fasted for 6 hours, were anaesthetized using Sodium Pentobarbital (Lethabarb,) at an initial dosage based on the weight of the animal (heterozygous 1.3µL/g, homozygous 1µL/g). Pedal reflexes were checked to ensure the animals was non-responsive to pain. Collection of tissues, following protocol (see appendix 2) was undertaken before opening the chest cavity. Blood collected as per: Blood sample preparation’.

After Terminal

Blood sample preparation

Blood collected at the terminal time point was collected from the inferior vena cava, transferred to heparinised blood collection tubes (BD Vacutainer), slightly agitated (10 seconds) and allowed to stand at room temperature for 6 minutes. Remaining blood was collected with a syringe from the chest cavity, and immediately transferred to a 1.5mL Eppendorf tube. Samples were then centrifuged at 2000 g for 10 minutes at 4°C. Plasma and serum was then aliquoted and frozen with liquid nitrogen before storing at -80 °C for later analysis. Blood analysis for the current thesis was undertaken using serum samples only. Samples were diluted (1:1/1:3) with MilliQ water prior to enzymatic assay.

Bile Sample preparation

Bile samples were thawed at room temperature and immediately diluted in MilliQ water (1:3) for biochemical analysis. Analysis of biliary bile acids were undertaken in bile samples diluted 1:50 in MilliQ water.

Preparation of frozen liver sample

Frozen liver tissue was first homogenized in a pre-cooled (liquid nitrogen) mortar and pestle, to a fine pale pink powder, ensuring tissue remained frozen by adding small amounts of liquid nitrogen. Tissue was homogenized until it reached a fine, pale pink powder. ~100mg of frozen liver tissue was then transferred into a 15mL falcon tube and stored at -80°C for later lipid extraction.

Preparation of faecal sample

Faeces collected from metabolic cages were weighed fresh, and then dried for 48 hours and stored in an air-tight container until later analysis. Entire dried samples were homogenized using a mortar and pestle, until it reached a fine powder. At time of analysis, ~100mg samples were aliquoted into 15mL falcon tubes for later lipid extraction.

Extraction of lipid from tissue/faeces

Isopropyl-alcohol (50mg/mL) was added to cold pre-weighed tissue samples (see Preparation of frozen liver tissue) and vortexed for 1-minute ensuring that all tissue was submerged in the solvent. The tissue was left to stand for 10 minutes, then sonicated without heat for 10 minutes. After sonication, the sample was centrifuged (43000 g, 20°C, 10 minutes) and the supernatant transferred into a separate falcon tube. A constant amount (500µL) of supernatant was transferred into a 1.5mL Eppendorf tube and evaporated using a rotary evaporator (Labogene Scanspeed 40) at 35°C. 250µL of isopropanol was added to resuspend the sample and vortexed for 2 minutes. Cholesterol and triglyceride concentration of the samples were determined with the COBAS Integra 400 plus, using the total cholesterol (CHOL2, Roche Diagnostics, Australia) and triglycerides (TRIGL, Roche Diagnostics, Australia) assay kits respectively.

Biochemical Analysis

Analysis of metabolites and lipids were tested using enzymatic, colorimetric assays, using the COBAS Integra 400+ (Roche Diagnostics, Australia). All reagent cassettes suitable for the COBAS Integra, were sourced from Roche Diagnostics. Additional analytes (phospholipids, bile acids) were analysed using commercial kits (Phospholipids C, Wako; Total Bile Acids, Roche Diagnostics) that were programmed according to instructions, for analysis in the COBAS after optimisation. All commercially available biochemical analysis kits were verified with their appropriate calibrators and quality control material prior to analysis of samples (Calibrator for automated systems, Precinorm ClinChem Multi 1 and 2; Roche Diagnostics, Australia).

Calculation of Biliary ratios

Analysis of biliary *cholesterol: phospholipid* concentration was determined by conversion of units (mg/dL to mmol/L) , based on the molecular weight of phosphatidylcholine (MW:786.129). *Chol:phos ratio* = cholesterol: phospholipid (mmol/L). *Lipid: BA ratio* = Lipid (cholesterol + phospholipid): Bile acid

Statistical Analysis

Animals were subject to the same protocol and analysed based on identification number and were therefore analysed statistically as independent samples respective of their ID numbers. When analysing the whole cohort, data amongst all relevant animals were combined (i.e. independent of sex) and analysed collectively, including assessment for gaussian distribution. When animals were analysed specific to sex, the relevant datasets were split based on the sex of the animal, and analysed independently of each other, also including assessment of normality. Normality was confirmed in animals with skewness and kurtosis factors greater than -3 and less than +3. Two-tailed, parametric (unpaired t-test) or nonparametric (Mann Whitney U-test) tests were used to compare Gunn and control groups with and without gaussian distribution, respectively. For data produced in duplicate (blood, bile, hepatic lipid content and faecal lipid content parameters) data were averaged. Bivariate analysis of variables was tested using Pearson's or Spearman's correlation. A value of $p<0.05$ was used to determine significance. Results are either expressed as mean ± SD, or median (25–75% interquartile range).

All data was analysed statistically, using SPSS 24, and figures generated using PRISM Graphpad 7.0.

4.0 Results

Animal weight

To assess the potential impact of the Gunn phenotype and hyperbilirubinaemia on body mass and weight gain, fasting body weight measurements were recorded at the terminal timepoint, following fasting and compared.

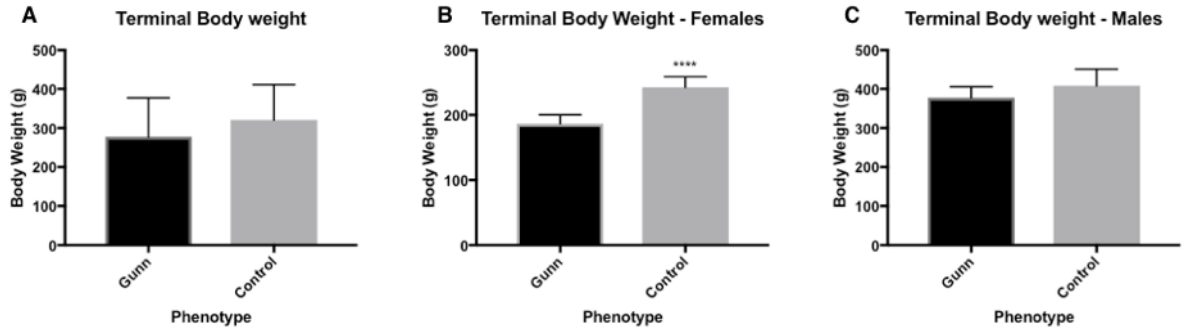


Figure 4.1 *A. Terminal body weight in all Gunn (n=19) and control (n=17) animals; B. female animals only [Gunn (n=10) and control (n=9)]; and C. male animals only [Gunn (n=9) vs. control (n=8)]. Data represented as mean ± SD *p<0.05, **p<0.01 ***p<0.001, comparison between Gunn and control animals.*

Weight gain over 21 days

Weight gain over a constant period of time was measured to highlight potential developmental differences in rate of growth, giving insight into development differences between Gunn rats and controls.

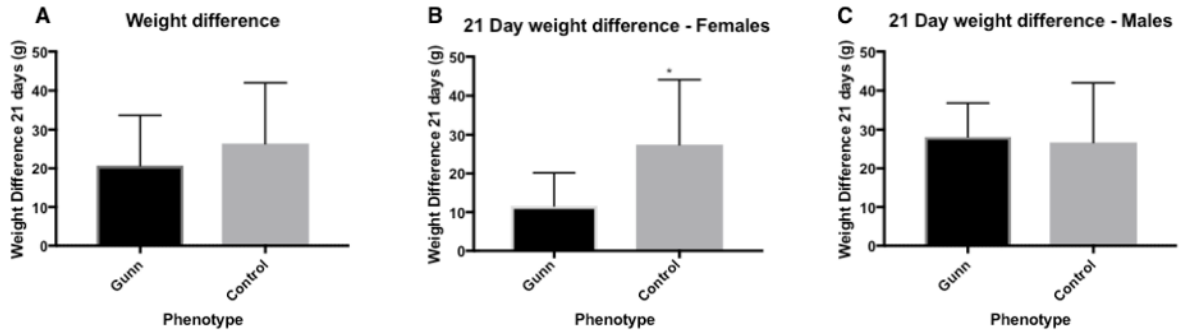


Figure 4.2 *Body weight gain in Gunn (n=19) vs. control (n=17) animals over 21 days. Body weight, measured on day -4 was subtracted from body weight measured on day 17 and averaged amongst phenotypic groups. The average difference in body weight (g) over 21 days of A. The whole cohort, B. female animals only (Gunn (n=10) vs. control (n=9) and C. male animals only Gunn (n=9) vs. control (n=8). Data represented as mean ± SD. Statistical significance represented as *p<0.05, **p<0.01 ***p<0.001, comparison between Gunn and control animals.*

Analysis of the entire cohort showed no significant difference ($p>0.05$) in body weight between Gunn and control animals. No significant difference ($p>0.05$) in body weight was determined in male animals (Figure 4.1C). Body weight was significantly reduced in female Gunn rats, compared to control animals (Figure 4.1B). Female Gunn rats only, demonstrated reduced weight gain over the period of investigation ($p<0.05$) compared to controls (Figure 4.2B), whereas no significant difference was observed in males ($p>0.05$).

Weekly Body Weight

Body weight of the animals was measured to outline the progression of weight gain in all animals.

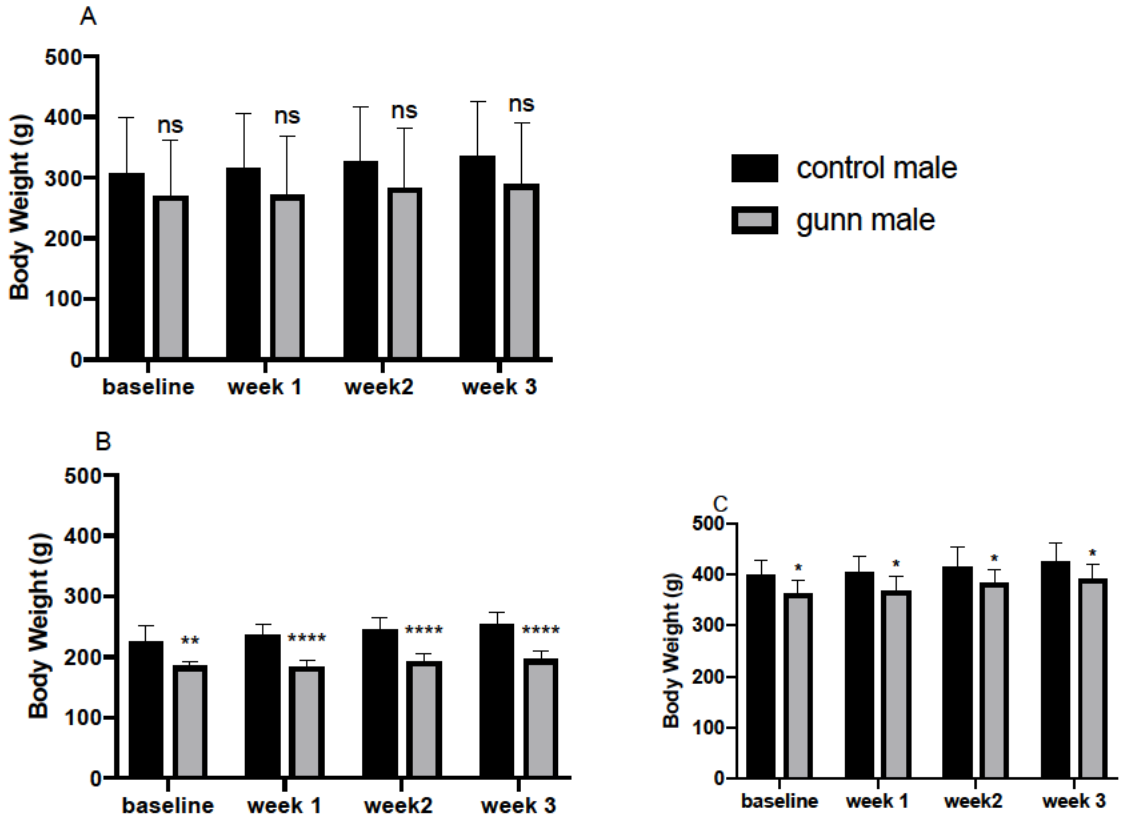


Figure 4.3 *Weekly body weight measurements in Gunn (n=19) vs. control (n=17) animals over 21 days. Body weight, measured 7 days apart, beginning at day -4 in the experimental protocol. The average weekly body weight (g) over 21 days of A. The whole cohort, B. female animals only (Gunn (n=10) vs. control (n=9) and C. male animals only Gunn (n=9) vs. control (n=8). A two-way ANOVA (time, bodyweight) analysis was performed. Data represented as mean ± SD. Statistical significance represented as *p<0.05, **p<0.01 ***p<0.001, comparison between Gunn and control animals.*

Figure 4.3 is a representation of bodyweight at weekly intervals through the experimental protocol. Amongst the whole cohort, Gunn rats had a lower body weight, compared to controls to a non-significant degree ($p>0.05$). Sex specific analysis showed a significantly decreased

bodyweight in female Gunn Rats, compared to female Controls ($p<0.001$). The male rats showed a similar trend, however, this was not significant.

Feed efficiency

Feed efficiency was calculated to determine the contribution of caloric intake to the overall weight gain. This further supports the potential association of decreased bodyweight to the Gunn rat phenotype.

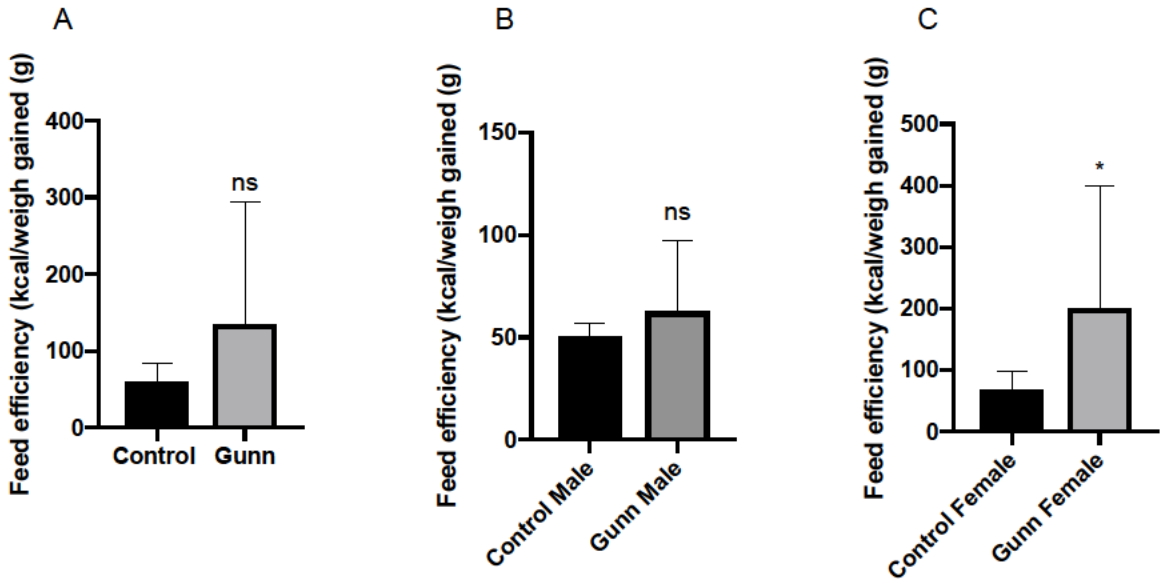


Figure 4.4 *Feed efficiency of Gunn (n=19) vs. control (n=17) animals over 21 days. Body weight gain determined as specified in Figure 4.1. Caloric intake depicted as 'average food intake * 3.1 (See appendix 1)'. The feed efficiency (g) over 21 days of A. The whole cohort, B. male animals only (Gunn (n=9) vs. control (n=7) and C. female animals only Gunn (n=10) vs. control (n=9). Comparison of means undertaken by Student t test/Mann- Whitney U test for normal and non-normal data, respectively. Data represented as mean ± SD. Statistical significance represented as *p<0.05, **p<0.01 ***p<0.001, comparison between Gunn and control animals.*

Non-significant differences in feed efficiency was observed in the whole cohort. Sex specific analysis of feed efficiency observed non-significant differences in the male cohort. Females, however, displayed a significantly increased caloric requirement, in order to gain bodyweight ($p<0.05$)

Metabolic cage analysis

Cumulative results from metabolic cage investigations over four, 24-hour time points, were measured to identify possible differences in the food and water intake in addition to faecal and urinary excretion.

Table 4.1 – Food and Water Consumption and Urine and Faecal Output in Gunn Rats

Variable	Control	Gunn	P value
Food Intake (g/day)	22.8 ± 4.31	20.6 ± 5.01	0.180
Male	25.9 ± 4.16	25.3 ± 2.18	0.708
Female	20.0 ± 1.84	16.4 ± 2.06	0.001**
Water Intake (g/day)	31.5 ± 9.68	29.5 ± 7.92	0.502
Male	32.4 ± 9.49	31.8 ± 7.99	0.876
Female	30.7 ± 10.4	27.8 ± 7.71	0.453
Urine output (g/day)	20.4 ± 8.52	18.6 ± 6.78	0.510
Male	19.9 ± 6.86	21.1 ± 5.91	0.697
Female	20.8 ± 10.1	16.4 ± 7.03	0.291
Faecal output (g/day:dry weight)	5.51 ± 1.22	5.22 ± 0.92	0.411
Male	6.08 ± 0.87	5.29 ± 1.09	0.123
Female	5.01 ± 1.30	5.15 ± 0.79	0.776

Table 4.1 *Food, water consumption and faecal output data Gunn (n=19) vs. control (n=17). Female animals only; Gunn (n=10) vs. control (n=9). Male animals only; Gunn (n=9) vs. control (n=8). Metabolic cage data collected over a period of 24 hours, at 4 timepoints (day -1, day 10, day 14 and day 18), and analysed collectively. Food intake (g), water intake (g), urinary output (g) and faecal output (dried) (g). Data presented as mean ± SD. Significance represented as *p<0.05, **p<0.01 ***p<0.001, comparison between Gunn and control animals.*

Table 4.1 shows that no significant differences existed in metabolic cage derived data, with one exception being food consumption in female Gunn animals, which was significantly reduced compared to controls ($p<0.01$).

Adult Blood Biochemistry

Table 4.2 represents the concentrations of biochemical constituents in plasma samples, comparing Gunn rats to control rats. Plasma samples were collected and stored as per experimental protocol at the terminal time point, following anaesthetisation and cannulation of the bile duct.

Table 4.2 – Adult Blood Biochemistry

Variable	Control	Gunn	P value
Total bilirubin (µmol/L)	2.21 ± 1.28	84.3 ± 26.4	p<0.001***
Male	2.29 ± 1.02	109 ± 15.04	p<0.001***
Female	2.13 ± 1.54	64.8 ± 13.8	p<0.001***
Direct bilirubin (µmol/L)	1.38 ± 0.88	7.52 ± 1.29	p<0.001***
Male	1.24 ± 0.61	8.04 ± 0.98	p<0.001***
Female	1.5 ± 1.08	7.11 ± 1.40	p<0.001***
Total cholesterol (mmol/L)	1.56 ± 0.28	0.98 ± 0.44	p<0.001***
Male	1.56 ± 0.23	1.41 ± 0.15	0.139
Female	1.56 ± 0.34	0.60 ± 0.12	p<0.001***
HDL (mmol/L)	1.36 ± 0.23	0.72 ±0.61	p<0.001***
Male	1.33 ± 0.22	1.37 ± 0.16	0.703
Female	1.36 ± 0.25	0.20 ± 0.09	p<0.001***
Triglycerides (mmol/L)	0.96 ± 0.6	1.08 ± 0.44	0.504
Male	1.04 ± 0.56	1.19 ± 0.52	0.586
Female	0.87 ± 0.66	0.98 ± 0.37	0.704
Phospholipids (mg/L)	123 ± 32.9	110 ± 21.5	0.874
Male	115 ± 36.9	129 ± 12.9	0.357
Female	130 ± 29.2	94.0 ± 11.9	0.016*
Total bile acids (µmol/L)	18.8 ± 17.3	39.0 ± 43.8	0.92
Male	21.6 ± 24.7	35.6 ± 60.7	0.56
Female	16.3 ± 7.34	41.7 ± 27.14	0.017**

Table 4.2 *Plasma biochemistry. 13wk old Gunn vs. control rats. Gunn (n=17) vs. control (n=19). Females animals only; Gunn (n=10) vs. Control (n=9). Male animals only; Gunn (n=9) vs. control (n=8). Data represented as mean ± SD *p<0.05, **p<0.01 ***p<0.001, comparison between Gunn and control animals*

Measurement of total bilirubin in the whole cohort confirmed the state of hyperbilirubinemia in Gunn rats, which was almost 20-fold greater than the control animals ($p<0.001$). Highly significant differences in total bilirubin existed in both male and female animals compared to controls. Amongst all animals, Gunn rats displayed a greater concentration of direct (conjugated) bilirubin compared to control rats ($p<0.001$).

Total serum cholesterol concentration was measured to assess the lipid profile of the animals in question. Analysis of the whole cohort revealed that Gunn rats had reduced (~33%) circulating cholesterol concentrations, compared to controls ($p<0.001$). Sex specific analysis showed an approximate 80% reduction in the female cohort ($p<0.001$). Conversely, male animals demonstrated no significant difference ($p>0.05$). HDL concentrations in Gunn rats were significantly reduced, compared to controls ($p<0.001$), and even more pronounced in the female cohort ($p<0.0001$), with an approximate 85% reduction in Gunn rats, compared to controls.

Plasma triglycerides were measured to further determine the lipid profile of animals. No significant difference in the whole cohort or within sex specific groups was noted ($p>0.05$).

Significant differences in plasma phospholipids, existed in the female cohort only, showing that Gunn rats had (28%) reduced concentrations, when compared to controls ($p<0.05$). Female Gunn rats had a significantly greater plasma bile acid concentrations, compared to female controls ($p<0.05$).

Bile biochemistry – Biliary concentration

Concentrations of biliary constituents were measured to determine the composition of bile, which allowed for analysis of concentration-dependent determinants of biliary lipid secretion.

Table 4.3 – Biliary concentration

Variable	Control	Gunn	P value
Total bilirubin (µmol/L)	107 ± 21.2	28.9 ± 4.95	<0.001***
Male	116 ± 22.4	32.1 ± 3.3	<0.001***
Female	97.7 ± 16.8	25.0 ± 3.69	<0.001***
Direct bilirubin (µmol/L)	88.1 ± 32.1	22.0 ± 3.62	<0.001***
Male	105 ± 37.2	24.2 ± 3.22	0.003**
Female	71.7 ± 15.5	19.5 ± 2.22	<0.001***
Phospholipids (mg/L)	171 ± 52.3	206 ± 56.8	0.138
Male	142 ± 26.4	168 ± 47.2	0.262
Female	200 ± 57.4	252 ± 23.4	0.095
Cholesterol (mmol/L)	0.15 ± 0.05	0.16 ± 0.07	0.499
Male	0.14 ± 0.04	0.11 ± 0.03	0.272
Female	0.15 ± 0.07	0.22 ± 0.04	0.073
Total bile acids (µmol/L)	9845 ± 2411	9508 ± 3761	0.799
Male	8279 ± 1374	8131 ± 3631	0.927
Female	11411 ± 2240	11159 ± 3554	0.889
chol:phos ratio	69.1	63.4	0.512
Male	78.75	58.4	0.928
Female	59.6	69.5	0.188
lipid:BA ratio	0.016	0.018	0.221
Male	0.018	0.017	0.863
Female	0.013	0.02	0.004**

Table 4.3 *Bile biochemistry. Biliary output in 13 week Gunn vs. control animals. Whole cohort; Gunn (n=11) vs. control (n=12). Females animals only; Gunn (n=5) vs. control (n=6). Male animals only; Gunn (n=6) vs. control (n=6). Chol:phos ratio = cholesterol: phospholipid ratio. Lipid: BA ratio = Lipid (cholesterol + phospholipid): Bile acid ratio. Data represented as mean ± SD *p<0.05, **p<0.01 ***p<0.001, comparison between Gunn and control animals.*

Table 4.3 presents the concentrations of biliary constituents, and analysis of lipid composition, relative to bile acids. Total bilirubin and direct bilirubin concentrations were significantly decreased in Gunn rats, compared to controls, amongst all analysis groups ($p<0.01$). Increases in cholesterol and phospholipid concentrations were present in the female Gunn rats, however, these only neared significance ($p>0.05$). Ratios generated by comparing 1. Cholesterol concentration and phospholipid concentration 2. lipid (cholesterol + phospholipid) concentration to bile acid concentration. Analysis of the chol:phos ratio revealed non-significant differences amongst all analysis groups ($p<0.05$). Female Gunn rats had significantly increased lipid:BA ratios, compared to control ($p<0.01$). No significant difference was observed in males.

Bile biochemistry - Relative secretion

Relative secretion rates of bile were determined to identify differences in calculated relative biliary excretion, where factors such as bodyweight and total bile flow were accounted for.

Table 4.4 – Relative Biliary Secretion

Variable	Control	Gunn	P value
Bile flow mL/hr/100g	0.46 ±0.07	0.57 ± 0.17	0.057
Male	0.44 ± 0.07	0.47 ± 0.12	0.506
Female	0.48 ± 0.06	0.69 ± 0.14	0.022*
Total bilirubin (nmol/hr/100g)	48.4 ± 7.76	16.0 ± 3.67	< 0.001***
Male	50.1 ± 9.19	15.0 ± 3.67	< 0.001***
Female	46.7± 6.39	17.3 ± 3.7	< 0.001***
Direct bilirubin (nmol/hr/100g)	43.2 ± 12.8	11.0 ± 2.43	< 0.001***
Male	49.0 ± 9.40	14.7 ± 3.66	0.001**
Female	34.1 ± 5.41	13.5 ± 3.16	< 0.001***
Phospholipids (mg/hr/100g)	0.80 ± 0.33	1.21 ± 0.57	0.045*
Male	0.62 ± 0.16	0.76 ± 0.18	0.168
Female	0.99 ± 0.36	1.75 ± 0.32	0.006**
Cholesterol (nmol/hr/100g)	68.0 ± 32.7	100 ± 55.9	0.109
Male	62.4 ± 25.8	57.1 ± 24.4	0.753
Female	73.7 ± 40.0	153.3 ± 27.7	0.006**
Total bile acids (µmol/hr/100g)	4.58 ± 1.58	5.37 ± 2.47	0.371
Male	3.58 ± 0.57	3.53 ± 0.71	0.976
Female	5.47 ± 1.66	7.34 ± 1.83	0.1

Table 4.4 *Bile biochemistry. Relative secretion at in 13 week Gunn vs. controls. Whole cohort: Gunn (n=11) vs. control (n=12). Females animals only; Gunn (n=5) vs. control (n=6.) Male animals only Gunn (n=6) vs. control (n=6). Assumed relative secretion of named parameters based on information (body weight, biliary flow) recorded at time of tissue collection. Data calculated accounts for bodyweight and bile flow. Data represented as mean ± SD. Significance represented as *p<0.05, **p<0.01 ***p<0.001, comparison between Gunn and control animals*

Table 4.4 represents excretion of biliary constituents, relative to bile flow and body weight. Volumes of bile secreted in a 30-minute period were used to determine bile flow (per hour, relative to animal weight), and excretion of biliary constituents. Analysis of the entire cohort showed increased bile flow in Gunn rats, nearing significance ($p>0.05$). Analysis of sex specific groups showed no significant difference in male bile flow ($p>0.05$), whereas females displayed a significantly greater bile flow in Gunn rats, compared to controls ($p<0.05$).

Relative bilirubin excretion through the bile was measured, and represented as a rate of excretion, relative to the body weight. Total bilirubin, and direct bilirubin excretion was significantly reduced in Gunn rats, compared with controls ($p<0.001$). Sex specific analysis revealed the same outcomes ($p<0.05$) in both total bilirubin and direct bilirubin analyses.

Relative biliary secretion of phospholipids was increased in Gunn rats compared to controls, nearing significance ($p>0.05$), when the whole cohort was analysed. When the male group was analysed, the same trend occurred, however, did not reach significance ($p>0.05$). In the female cohort, Gunn rats excreted significantly more phospholipids into the bile, than female controls ($p<0.01$), relative to body weight.

Analysis of all animals presented a non-significant increase in cholesterol secretion in Gunn rats ($p>0.05$). Similarly, to the analysis of phospholipids, the female specific data shows a highly significant difference in cholesterol secretion between Gunn and control animals ($p<0.05$).

Total bile acids did not differ significantly when the whole cohort was analysed ($p>0.05$). The analysis of female bile acid excretion showed that Gunn animals secreted more bile acids, than

female controls however, this difference was not statistically significant (p>0.05). The relative total bile acid excretion did not differ in the males (p>0.05).

Hepatic cholesterol content

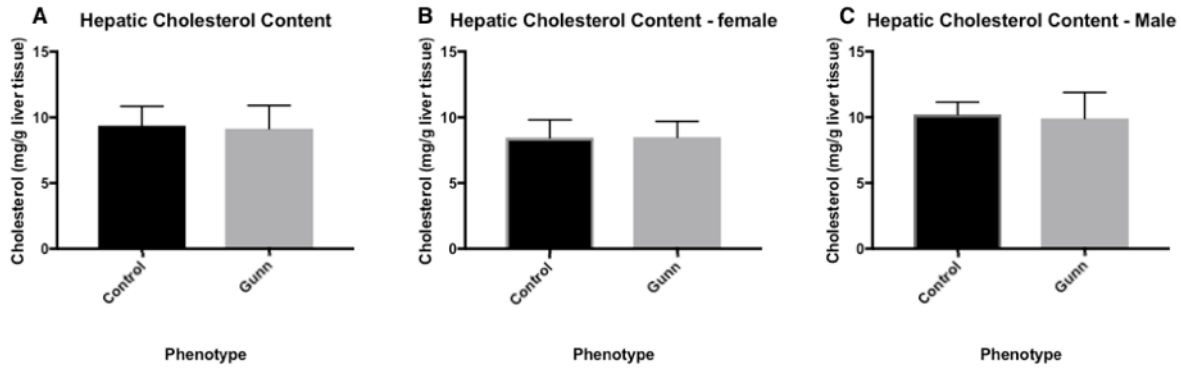


Figure 4.5 *Hepatic cholesterol content of hepatic tissue obtained from the ventral lobe. Tissue collected under non-hypoxic conditions and extracted using isopropanol. A. Hepatic cholesterol content in the whole cohort B. Hepatic cholesterol content in the female cohort C. Hepatic cholesterol content in the male cohort. Data represented as mean ± SD expressed in mg cholesterol per gram of hepatic tissue (mg chol/g tissue). Significance represented as *p<0.05, **p<0.01 ***p<0.001, comparison between Gunn and control animals.*

Figure 4.5 shows that hepatic cholesterol content between the Gunn and controls, did not differ when the whole cohort was analysed (p>0.05). Sex specific analysis found similar results, where hepatic cholesterol content, did not differ between Gunn and control animals in both the male (p>0.05) and female (p>0.05) groups.

Faecal cholesterol content

Figure 4.6 shows the amount of cholesterol that is excreted per day, based on extraction of faecal cholesterol and analysed relative to the mass of faeces excreted over a period of 24 hours.

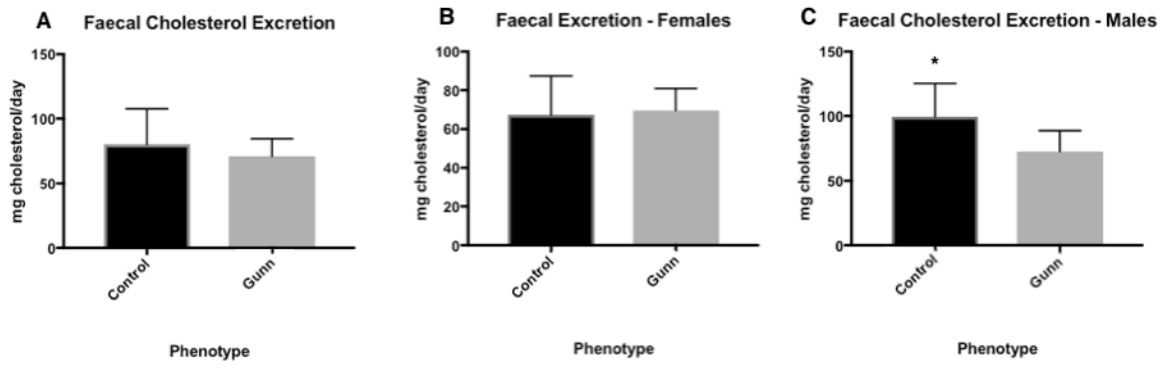


Figure 4.6 *Faecal cholesterol excretion. Quantification of cholesterol extracted from homogenized dried faecal samples (normalised to 24 hour collection). Faecal cholesterol content in the whole cohort; Gunn (n=17) vs. control (n=19). B. Faecal cholesterol content in the female cohort; Gunn (n=10) vs. control (n=9). C. Faecal cholesterol content in the male cohort; Gunn (n=9) vs. control (n=8). Faeces collected over a period of 24 hours on day 14 of the study and dried for 48 hrs before storage at room temperature. Data represented as mean ± SD expressed in mg cholesterol excreted per day. Figures generated from faecal cholesterol excretion from Day 14. *p<0.05, **p<0.01 ***p<0.001, comparison between Gunn and control animals.*

Daily faecal cholesterol excretion was determined based on samples obtained from day 14 metabolic cage data. Analysis of the whole cohort showed no significant difference in the excretion of faecal cholesterol per day. Only the male cohort showed a significant increase in faecal excretion in controls, compared to Gunn rats ($p<0.05$).

Fractional *de novo* cholesterol synthesis

To identify possible differences in endogenous cholesterol synthesis between Gunn rats and controls, fractional *de novo* cholesterol synthesis was determined and is presented in Figure 4.7

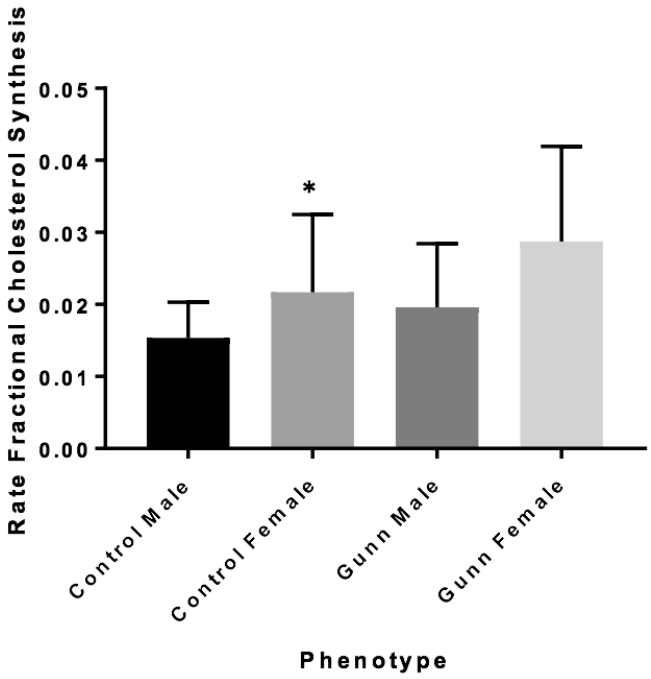


Figure 4.7 *Rate of fractional cholesterol synthesis. Represented as rate increase of percentage contribution C ^{13}-cholesterol to total cholesterol, per unit time. Control male n=8, control female n=9, Gunn male n=9, Gunn female n=10. Data represented as mean ± SD *p<0.05, comparison between Gunn and control animals, of same sex.*

Contribution of heavy labelled cholesterol to total cholesterol pool is represented as a percentage of total cholesterol, derived from analysis of blood spots. The increase of this percentage per unit time is calculated to determine the rate of fractional cholesterol synthesis. Female Gunn rats demonstrated significantly elevated fractional cholesterol synthesis compared to their respective controls ($p<0.05$), whereas no difference was seen in males ($p>0.05$).

Juvenile blood biochemistry

Table 4.5 shows the plasma biochemistry of juvenile rats. Analysis of juvenile animals provides a model that is independent of influence associated with sexual maturity and hormonal development.

Table 4.5 – Juvenile Blood Biochemistry

Variable	Control	Gunn	P value
Total bilirubin (µmol/L)	2.28 ± 1.21	77.3 ± 32.3	<0.0001***
Male	2.29 ± 1.53	72.63 ± 32.00	<0.0001***
Female	2.28 ± 0.53	83.9 ± 34.2	0.001**
Total Cholesterol (mmol/L)	1.54 ± 0.62	1.19 ± 0.31	0.071
Male	1.60 ± 0.76	1.21 ± 0.30	0.181
Female	1.45 ± 0.32	1.17 ± 0.34	0.184
HDL (mmol/L)	1.14 ± 0.38	0.86 ± 0.27	0.025*
Male	1.24 ± 0.43	0.89 ±0.25	0.067
Female	0.99 ± 0.24	0.82 ± 0.32	0.346
Triglycerides (mmol/L)	0.92 ± 0.21	0.94 ± 0.42	0.822
Male	0.93 ± 0.27	0.89 ± 0.32	0.724
Female	0.89 ± 0.08	1.04 ± 0.55	0.508
Phospholipids (mg/L)	150 ± 31.8	118 ± 23.1	0.004**
Male	156 ± 38.0	120 ± 21.6	0.022*
Female	141 ± 7.87	115 ± 26.7	0.078
Total bile Acids (µmol/L)	122 ± 79.7	110 ± 66.0	0.657
Male	100 ± 72.7	93.2 ± 61.3	0.823
Female	156.8 ± 86.0	134 ± 69.3	0.626

Table 4.5 *Plasma biochemistry of 3 week-old Gunn vs. controls. Gunn (n=17) vs. control (n=13). Females animals only; Gunn (n=8) vs. control (n=5) Male animals only; Gunn (n=10) vs. control (n=7). Samples data represented as mean ± SD. Significance represented as *p<0.05, **p<0.01 ***p<0.001 comparison between Gunn and control animals.*

Serum bilirubin concentrations differed significantly between Gunn and control rats, where controls were 3% that of Gunn rats, when analysing plasma bilirubin concentration ($p<0.001$). Total cholesterol concentration decreased in male and female Gunn rats by ~25%, and ~20% respectively; however, these differences were not statistically significant ($p>0.05$). Analysis of the whole cohort revealed a significant reduction in HDL in Gunn rats; approx. 75% that of the controls ($p<0.05$*) where a ~30% reduction was reported in males, nearing significance ($p>0.05$).

Gunn rats presented a ~20% reduction in plasma phospholipids ($p<0.01$), compared to controls. Sex specific analysis revealed that male Gunn rats had a significantly reduced ($p<0.05$) concentration compared to controls (~25%). Females, on the other hand, showed the same trends, but the difference did not reach statistical significance (~20%). Analysis of total bile acids did not reveal any significant differences between Gunn rats and controls ($p>0.05$).

Analysis of blood biochemistry showed significant differences between adult and juvenile animals. Adult males (Table 4.2) showed markedly increased total bilirubin concentrations (109 μM), compared to juveniles (Table 4.5) (72 μM). Females on the other hand, showed a notable increase in total bilirubin in juveniles (84 μM) compared to adults (65 μM). Cholesterol concentrations in female adult Gunn rats (Table 4.2) were significantly lower (51%) than in juveniles (Table 4.5).

Adult Gunn rats displayed a 52% increase in bile acids compared to controls (Table 4.2). However, juvenile Gunn rats had a 10% reduction in bile acids compared to controls (Table 4.5). Interestingly, the juvenile animals displayed a significantly increased concentration of

plasma bile acids, compared to adults when comparing concentration across both Gunn and control animals.

5.0 Discussion

5.1 Summary of Findings

The main findings of this thesis were that female Gunn rats possess an increased fractional rate of endogenous cholesterol synthesis and biliary lipid secretion. Throughout the entire study, the effects of UGT1A1 mutation and/or hyperbilirubinaemia were more significant in females, inferring a sex specific effect. Perturbation of endogenous synthesis and biliary excretion could potentially explain the alteration of lipid profile in hyperbilirubinaemic humans with Gilbert's syndrome, and in Gunn rats.

The primary aim of the current study was to determine the rate of endogenous cholesterol synthesis in hyperbilirubinaemic Gunn and littermate normobilirubinaemic, heterozygote control rats (Figure 4.5). Increased fractional cholesterol synthesis was observed in female Gunn rats compared to female controls. However, no difference was observed between male Gunn and control groups. Profound hypocholesterolaemia was observed in female Gunn rats, with a concurrent increase in plasma bile acids (Table 4.2), with less pronounced increases in males. It was also hypothesised, that Gunn rats would demonstrate increased biliary lipid secretion (Table 4.3-4), compared to controls, supporting the hypocholesterolaemic phenotype observed previously [81, 82, 136, 144]. Uncoupling of biliary lipid (cholesterol + phospholipid) and bile acid (BA) secretion was present, as evidenced by a significantly increased lipid:BA ratios in female Gunn rats, with less prominent effects observed in males (Table 4.3). Despite this, relative faecal cholesterol excretion did not differ between Gunn and control animals

(Figure 4.6), suggesting that enhanced cholesterol secretion was accompanied by increased reabsorption from the gut.

A secondary aim was to identify and characterise sex specific differences in lipid metabolism, in Gunn rats and controls. To further test the possibility of sexual dimorphism, juvenile Gunn and control rat plasma was tested and revealed similar blood bilirubin, total cholesterol, and triglyceride concentrations (Table 4.5). Interestingly, juvenile male Gunn rats had greater decreases in HDL and phospholipids, compared to juvenile female Gunn rats, despite not reaching significance. Review of literature revealed limited information regarding lipid status of Gunn rats, however the available results demonstrate weaker trends in hyperbilirubinaemic humans (GS individuals) and more pronounced effects in Gunn rats [81, 136]. These data suggest that sexual maturity of animals may influence the effect of hyperbilirubinaemia/UGT1A1 dysfunction on lipid metabolism.

Due to the limited knowledge in this field, characterisation of male and female Gunn rat circulating lipid status and cholesterol biosynthesis are critical to improving our understanding of UGT1A1 dysfunction/unconjugated hyperbilirubinaemia. Although previous studies have characterised circulating lipid status in Gunn rats [81, 82, 136, 144], this is the first study to determine the impact of UGT1A1 dysfunction on multiple contributors to cholesterol metabolism including endogenous synthesis, hepatic storage, biliary and faecal excretion, in littermate Gunn and control animals.

5.2 Confirmation of hyperbilirubinemia

The Gunn rat phenotype was determined at the time of weaning, with hyperbilirubinaemic animals displaying yellow colouration of the fur and the skin, compared to controls. Total

bilirubin concentrations (Table 4.2) were also assessed in blood with Gunn rats approximating 84 μM, similar to other studies (80 μM) [3] . Few other studies have explicitly outlined the serum bilirubin concentration relative to lipid status [81, 136], instead reporting the concentration of unconjugated bilirubin (UCB), instead of total bilirubin. Despite this difference in methodology the relationship between total bilirubin and lipid profile in current study agreed with previously published data using UCB concentrations.

The relative degree of hyperbilirubinemia was notably increased in the males (~50 fold increase versus heterozygotes) compared to the females (~30 fold increase), which could contribute to the modulation of lipid status, based on concentration dependent effects previously proposed [136] (Table 4.2). The current study, however, did not agree with a concentration dependent effect, because alteration of lipid status was more prominent in female animals who had lower bilirubin concentrations. This observation, therefore, argued against the idea of a concentration dependent effect of bilirubin in Gunn rats, inferring alteration of lipid status could be mediated indirectly by the cause of hyperbilirubinaemia (UGT1A1 deficiency) and potentially other targets of this enzyme (ie. oestrogen/testosterone).

5.3 Gunn animals have a reduced body weight compared to controls

Prior to addressing the key hypotheses of this thesis, phenotypic animal differences were compared between the groups. These results demonstrated reduced body weight in Gunn rats, which were supported by results of previous studies [81, 136] and in human studies of Gilbert's syndrome. The current study, however, observed significantly reduced body weight (~23%) in female Gunn rats only compared to the control females, with smaller, non-significant reductions in males (~8%) (Figure 4.1). Interestingly, analysis of feed efficiency (kcal required

to gain 1g body weight) showed in increased in caloric requirement in female Gunn rats, suggesting perturbed association between food intake and weight gain (Figure 4.4).

Human studies of Gilbert's syndrome have outlined potential negative relationships between body weight and circulating bilirubin concentrations [140, 146-148]. Interestingly, some studies have also reported no correlation [117, 149], and one study demonstrated a positive correlation [150]. This highlights the variability of human trials, especially when considering factors such as BMI, which accounts for both height and weight of individuals. Belo et.al [150] reported a positive correlation between BMI and total bilirubin in non-obese participants. Interestingly, obese participants displayed a negative correlation between body fat percentage and total bilirubin concentrations in the same study. Questions remain as to whether obesity influences bilirubin or whether bilirubin influences lipid and whole-body metabolism, strengthening the rationale for the study completed in this thesis.

Assessment of weight gain over time, allowed for the assessment of rate of growth in age matched Gunn/control animals. Female Gunn rats gained significantly less weight over a 21 day period compared to controls, suggesting that UGT1A1 dysfunction could impair growth (Figure 4.2). The female specific effects could result from inhibition of UGT1A1 mediated glucuronidation of UCB and oestrogen [151]. Although there is no evidence to support an effect of UGT1A1 dysfunction on circulating oestrogen in mammals, competitive inhibition between estradiol and unconjugated bilirubin has been observed in cell culture [151]. Interestingly, when oestrogen production is decreased in ovariectomised rats, food intake and rate of weight-gain increases significantly, the results of which support the findings presented here [151].

Although bodyweight alone does not explicitly account for differences in the size of animals, these findings contribute to the characterisation of the Gunn rat phenotype. However, additional contributing factors influence body weight including caloric intake and locomotion [152, 153] .

Therefore, food, water intake, urinary and faecal output were also assessed at 4 timepoints throughout the study (Table 4.1). Combining these data showed that female Gunn rats consumed significantly less food than controls, which could contribute to reduced body weight. Similar trends were observed in water intake and faecal/urinary output in the same animals, although they were not statistically significant (Table 4.1). Male Gunn rats, on the other hand, showed similar non-significant trends in food and water consumption. Faecal and urinary output, however, displayed the opposite relationship, with male Gunn rats excreting more faeces and urine than male controls. These data further suggest that reduced caloric intake by female Gunn rats contributes to their reduced weight gain and faecal output. These differences could also contribute to altered lipid status in Gunn rats, because caloric restriction alters plasma LDL concentrations [154], which is regulated by feedback control in mevalonate (and therefore cholesterol) synthesis [24].

Alterations in cholesterol transport are also observed in situations of increased dietary cholesterol intake [155] whilst maintaining a relatively unchanged LDL:HDL plasma cholesterol ratio. The chow diet used in this study contained negligible amounts of cholesterol (Appendix 1), eliminating confounding effects of altered dietary cholesterol intake. Conversely, UGT1A1 dysfunction could contribute to reduced body weight, resulting in reduced metabolic demand (ie. reduced need for UDP-glucuronic acid synthesis, conjugation and active transport of glucuronide conjugates in the bile). Therefore, it is speculated that reduced energy demand, would reduce the requirement for caloric intake in UGT1A1 mutant

animals. Assessment of whole-body metabolic rate using indirect calorimetry would provide clarity concerning whether Gunn rats consume less food, due to reduced metabolic demand.

Overall, the data suggests that dietary factors are unlikely to represent a key factor altering lipid status in Gunn rats. The heightened significance in female Gunn rats, which paralleled the sex specific trends in the following sections, suggests an effect that is dependent on sex, UGT1A1 deficiency and potentially elevated oestrogen concentrations.

5.4 Alteration of the plasma lipid status in Gunn rats

In this thesis, it was hypothesised that reduced serum lipid/cholesterol concentrations would be observed in Gunn rats. This hypothesis was supported only in female Gunn rats (Table 4.2). Assessment of lipid status is important because cholesterol and triglyceride concentrations are modifiable risk factors for cardiovascular disease and are also reduced in Gilbert's syndrome [8] , which might explain protection from CVD in this hyperbilirubinaemic cohort. Previous studies have also characterised circulating lipid status of hyperbilirubinaemic animals [136, 139, 144, 156]. This was the first study to simultaneously assess biliary lipid secretion (Table 4.3-4), and endogenous cholesterol synthesis in the Gunn rat, necessary for a more complete understanding of the hypocholesterolaemic effects noted. Importantly, the study design also allowed for combined assessment of age, sex specific and phenotypic effects, which is critical to understanding interactions between UGT1A1 and potential hormonal effects mediated by oestrogen, which UGT1A1 also conjugates [151].

Total cholesterol concentrations (Table 4.2) were reduced in the whole cohort of Gunn rats, however, significant differences only occurred in females. Previous studies have noted similar reductions in females [81, 136] and non-significant differences in males [81]. The percentage

reduction of HDL (86%), observed in Gunn females, closely reflects those of previous studies (87-89%) [81]. Males on the other hand, showed only a ~10% decrease [136].

HDL contributes the greatest amount of cholesterol to total cholesterol concentrations in rats [157]. However, this is not the case in humans [156]. This effect in rats is caused by a deficiency of cholesteryl ester transfer protein (CETP) [156], which facilitates the uptake of dietary cholesterol and the transport of cholesteryl esters from HDL to apo B containing particles (VLDL and LDL) [157, 158]. It was therefore expected that HDL concentrations in rats closely reflected changes in total cholesterol. Human Gilbert's syndrome individuals demonstrate concurrent reductions in LDL and total cholesterol [136, 159], thereby arguing against HDL specific reduction in hyperbilirubinaemia. LDL concentrations were below the detection limits of the analysers used in this thesis, however, previous Gunn rat studies have reported reductions in plasma LDL and HDL [136, 139]. This suggested that the perturbation of cholesterol metabolism, observed in UGT1A1 deficient animals is not specific to particular lipoproteins, suggesting cholesterol synthesis might be reduced.

Additional evidence to support cholesterol specific effects include similarities in plasma triglyceride concentrations between Gunn and control rats, which are in agreement with previous findings [136, 139]. Only one study has reported significant reductions in Gunn rats, using a colony of animals derived from Europe [81]. These results are supported by studies that exogenously administered bilirubin to rats and observed alterations in lipid status [136]. These data support a role for bilirubin *per se* contributing to modulation of lipid status in Gunn rats.

This was the first study to document plasma bile acid and phospholipid concentrations, which were significantly increased in Gunn rats compared to controls (Table 4.2). Previous studies in

Esai hyperbilirubinaemic animals, who had conjugated hyperbilirubinemia, display similar serum bile acid concentrations [160], as presented in the current study.

5.5 Fractional cholesterol biosynthesis is increased in female Gunn rats

This thesis contains the first analysis of fractional cholesterol synthesis rates in Gunn rats, and revealed increased fractional biosynthesis rates in female Gunn rats, compared to controls (Figure 4.7). Increased endogenous synthesis rates have also been observed in animals administered bile acid sequestrants and statins [29, 30]. Interestingly, both studies reported overall reductions in plasma cholesterol. This data suggests that pharmacological inhibition of endogenous cholesterol production, stimulates *de novo* cholesterol synthesis as an adaptive, counter-regulatory response to maintain hepatic and circulating cholesterol concentrations. Similar increases in fractional synthesis rates, compared to these published studies, were observed in female Gunn rats, suggesting these animals were attempting to maintain hepatic stores, due to an unknown mechanism reducing hepatic cholesterol bioavailability. Interestingly, Li et al. [31] observed increased endogenous cholesterol synthesis in conditions of cytochrome P450 family 7 subfamily A member 1 (CYP7A1) overexpression, which could represent a mechanism to explain altered cholesterol metabolism in the current study.

5.6 Biliary lipid secretion is increased in female Gunn rats

It was hypothesised that biliary cholesterol excretion would be increased in Gunn rats, contributing to hypocholesterolaemia observed in these animals. In the female cohort, biliary cholesterol and phospholipid concentrations were increased by 32% and 20% respectively, nearing statistical significance (Table 4.3). Analysis of excretion relative to bile flow and body weight revealed significant increases in cholesterol (~52%) and phospholipid (~44%) secretion. Both sets of data indicated increased hepatic lipid excretion in female Gunn rats.

The degree of "coupled" biliary lipid (cholesterol and phospholipid) excretion was assessed by comparison of the cholesterol to phospholipid ratio (chol:phos) between phenotypes (Figure 4. 3). The current study observed unchanged chol:phos ratio when comparing Gunn and controls, inferring that coupled excretion of cholesterol to phospholipids was maintained in Gunn rats. The primary transporters responsible for the efflux of cholesterol and phospholipid across the canalicular membrane are ABCG5/8 [43] and ABCB4 (Mdr2) respectively[52]. Overexpression of ABCG5/8 resulted in a dramatic increase in biliary cholesterol, accompanied by a moderate increase in phospholipid content [161], the effects of which were reversed in ACBG5/8 knockout models [48]. ABCB4 knockout mouse models showed near complete inhibition of biliary phospholipid secretion, with minor alterations in cholesterol secretion, thereby dramatically increasing the chol:phos [52, 162]. The insignificant difference between Gunn and control chol:phos ratios seen in the current study, suggested that the observed increase in biliary lipids is not specific to lipid transporters, but is perhaps specific to bile salt mediated lipid secretion.

Intracanalicular bile salt concentration.

Early studies have outlined a positive correlation between biliary bile salt concentration, and biliary lipid secretion [127, 129]. Coupled lipid secretion occurs between biliary lipids (cholesterol and phospholipids) and biliary bile salts, analysed by the lipid:bile acid ratio[126]. In the current study, the lipid:BA ratio was significantly greater in female Gunn rats, compared to controls, which suggested uncoupling of bile acid mediated lipid secretion (Table 4.3). This effect could be mediated by increased cholesterol/phospholipid content of the liver, or perhaps reduced bile flow, which would provide greater time for extraction of lipids from the canalicular membrane[8].

Biliary concentrations of cholesterol and phospholipids in Gunn rats were greater in female Gunn rats compared to controls despite insignificant changes in bile acid concentrations (Table 4.3). When expressed relative to body mass and bile flow however, biliary bile acid output was increased (cholesterol ~52%, phospholipid ~45%) in female Gunn rats compared to controls.

Organic Anion Concentration

The unchanged chol:phos ratio and increased lipid:BA ratio suggested perturbation of biliary lipid secretion by means that are independent of bile salts (Table 4.3). Verkade et al. [126] proposed that the concentration of organic anions, which in the current study, referred primarily to the concentration of conjugated bilirubin in the bile, may have influenced bile flow and therefore, lipid excretion. A series of studies by Verkade and others [127, 128, 130, 131, 160, 163, 164] demonstrate the inhibitory effects of organic anions on the detergent properties of bile acids, thereby resulting in decreased lipid extraction from the canalicular membrane and subsequently, reduced lipid:BA ratio. Apstein [128] observed inhibition of biliary lipid excretion following infusion of bilirubin and/or other organic anions, therefore suggesting a negative correlation between biliary organic anion concentration and biliary lipid excretion (nmol/min). However, no conclusive evidence is available to indicate the impact of decreased biliary organic anions on biliary lipid secretion.

The current study reported a significant decrease in the biliary conjugated bilirubin (an organic anion) concentration, as a result of impaired UGT1A1 function. I propose that the reduced concentration of organic anions (conjugated bilirubin) in bile would result improved formation of mixed micelles, by bile salts [163]. This could, therefore, allow for increased extraction of cholesterol and phospholipids from the canalicular membrane, per unit of bile acid [126]. This

conclusion, however, does not consider the increased relative bile flow seen in female Gunn rats.

Bile flow

The current study observed increased bile flow (relative to animal mass and time) in Gunn females, compared to controls (Table 4.4). As the bile comprises of mostly water, it was inferred that alteration of bile flow is a reflection the water incorporation into the bile, by either passive or active transport[165]. Factors such as differences in food intake, diurnal variation, and most importantly, osmotic gradient (imparted by organic anions) contribute to variances in bile flow [165]. When considering osmotic gradient, the increased bile flow (relative to mass and time) seen in female Gunn rats suggested a greater concentration of compounds that would increase the influx of water into bile or is simply reflective of reduced mass in Gunn rats. Indeed, bile flows were not significantly different in Gunn rats, arguing against a role for altered bile flow in influencing lipid extraction (Table 4.4). Increased CYP7A1 activity results in increased hepatic bile acid synthesis [23], and therefore I speculate increased bile acid secretion. Increased BA secretion would be expected to increase water efflux and concurrently, increase bile flow. In the current study, relative secretion of biliary bile acids was increased (cholesterol ~52%, phospholipid ~45%) in female Gunn rats compared to controls. These data support a hypothesis of elevated bile acid induced influx of water, or bile salt dependent flow (BSDF)[22, 165], and increased absolute bile flow in female Gunn rats, which may have facilitated increased biliary cholesterol secretion.

5.7 Juvenile plasma biochemistry

Due to the apparent sexual dimorphism in circulating cholesterol observed in adult Gunn rats, blood biochemistry of 3 week old, prepubescent animals were assessed. Sprague-Dawley and

Wistar rats, experience sexual maturity at approximately 6 weeks of age [166]. The rationale for prepubescent analysis of male and female Gunn and control rats, rests with UGT1A1 conjugating both oestrogen [151] and bilirubin. Upon aging, it is possible that UGT1A1 mutant Gunn rats might experience greater oestrogen concentrations than controls, because its conjugation and therefore excretion is impaired, potentially influencing lipid status. Analysis of juvenile Gunn rats with their heterozygote littermates, provided a model that is theoretically, independent of hormonal influence (Table 4.5).

Plasma bilirubin concentrations in juvenile versus adult control animals did not differ (~2 μM). Adult male Gunn rats, however, showed markedly increased concentrations of total bilirubin (109 μM), compared to juveniles (72 μM). Adult Gunn females on the other hand, had a lower concentration of total bilirubin (65 μM) compared to juveniles (84 μM). This suggested that the degree of hyperbilirubinaemia was more significant in adult males compared to females. This agrees with human studies of Gilbert's syndrome, where onset of the hyperbilirubinaemic phenotype occurs after puberty [167]. Previous studies in juvenile Gunn rats demonstrate varying degrees of hyperbilirubinemia [94, 144], emphasising that differing gunn rats colonies may experience various genetic backgrounds and phenotypes.

Cholesterol concentrations in female adult Gunn rats (Table 4.2) were significantly lower (51%) than in juveniles (Table 4.5). These age-associated changes were absent in the male cohort. I speculate that the onset of oestrogen production following puberty, may contribute to increased cholesterol turnover, either promoting CYP7A1 induced bile acid synthesis from cholesterol [168] or facilitating degradation/inhibition of ABCA1 mediated cholesterol efflux [169]. Early studies on oestrogen supplementation in adult rat hepatocytes, demonstrates

suppression of cholesterol synthesis [170, 171], however, these results could not be replicated in vivo [170].

Adult Gunn rats displayed a 52% increase in bile acids compared to controls (Table 4.2). However, juvenile Gunn rats had a 10% reduction in serum bile acids compared to controls (Table 4.5). Interestingly, the juvenile animals displayed a significantly increased concentration of plasma bile acids, compared to adults. This could be due to the increased turnover of cholesterol to bile acids in juveniles[172], whereas in adult animals, a notable portion of cholesterol is converted to steroid hormones. In juvenile animals, the uptake clearance of BA's (Taurocholate) into isolated rat hepatocytes, mediated by NTCP was ~45% of that shown in mature animals (>5 weeks) [172]. Less uptake into the liver, combined with inactive steroid synthesis could potentially explain the increase in the serum BA concentration noted. Norlin et al. observed an increase in CYP7A1 mediated enzymatic activity between infancy and adolescence [173] in pigs. This suggests that serum bile acid concentrations are reduced by factors associated with sexual maturity.

An interesting direction of future study might be determination of serum cholesterol profile and biliary lipid secretion, in mature ovariectomized Gunn and control rats. This experiment would test whether removal of a key oestrogen source would influence lipid metabolism in the presence of UGT1A1 dysfunction.

5.8 Potential hormonal influence on plasma lipid status

A secondary aim of the current study highlighted potential sexual dimorphism in cholesterol metabolism in the Gunn rat. Comparison of control and Gunn rats revealed a highly significant difference in serum total bilirubin in both male and females. Despite this similar alteration of total bilirubin, significant reductions in serum cholesterol was only observed in female Gunn

rats (Table 4.2). Furthermore, no significant reduction if cholesterol was observed is either male or female juvenile rats (Table 4.5). This infers the involvement of sexual hormones in the altered lipid profile seen in Gunn rats. Both bilirubin and oestrogen are substrates of UGT1A1 [174] which facilitate conjugation prior to excretion from the body. Inhibition of UGT1A1 which is characteristic to Gunn rats [94, 112, 175] could cause an increase in circulating oestrogen in a similar manner to bilirubin, due to impaired conjugation. Previous research has observed alterations of cholesterol synthesis, and transport [170, 171, 176-178] when oestrogen is altered. It is therefore possible that an increase in oestrogen is affecting cholesterol metabolism indirectly, independent of the hyperbilirubinaemic conditions. Future analysis of serum oestrogen levels will allow for the exploration of hormone dependent effects on cholesterol metabolism in Gunn rats.

5.9 Faecal cholesterol excretion does not differ between control and Gunn rats

The current study revealed no difference in hepatic cholesterol content between Gunn and control rats, suggesting increased transport of cholesterol into bile, in the presence of increased endogenous synthesis in female Gunn rats. Overexpression of CYP7A1 (CYP7A1-*tg* mice) results in unchanged hepatic cholesterol content and demonstrates increased endogenous synthesis and bile acid secretion [31]. This study also documented increased biliary bile acid secretion in Gunn rats (Table 4.4), however, it is acknowledged that total (faecal) neutral sterol and bile acid excretion was not measured. This limitation is important because following biliary secretion, biliary lipids and bile acids are transported to the duodenum, where 90-95% are reabsorbed by the intestine [165]. The remaining cholesterol and bile acids are excreted via the faeces; therefore analysis of faecal neutral sterols and bile acids is critical to understanding whether increased cholesterol/bile acid synthesis is accompanied by increased faecal loss, resulting in no net change in the whole body cholesterol pool. Previous studies have observed an increase in faecal cholesterol excretion when endogenous synthesis is increased [30]. The

current study assessed the cholesterol content of the faeces and found no significant difference between Gunn and control rats (Figure 4.6), however, overexpression of CYP7A1 [31] dramatically increases faecal bile acid content and could account for increased fractional cholesterol synthesis rates in female Gunn rats if CYP7A1 expression is confirmed.

5.10 Hypothetical theories to explain perturbed endogenous synthesis in hyperbilirubinaemic Gunn rats

The data contained in this thesis are not sufficiently comprehensive to make firm conclusions regarding the cause of altered lipid metabolism in female Gunn rats. Therefore, existing literature in the field has been used to provide a more comprehensive understanding of potential mechanisms of action. The following section outlines two hypothetical mechanisms which could explain the results observed.

5.92 Proposal 1: Upregulation of CYP7A1 increases cholesterol turnover to bile acid synthesis

One potential instigating factor for the observed effect in Gunn rats, may include the impact of hyperbilirubinaemia on hepatic expression of P450 cytochromes. It is well documented that hyperbilirubinemia in Gunn rats increases the expression of CYP1A1/1A2 via aryl hydrocarbon receptor agonism [179] [180] [181] which modulates the expression of other cytochromes that can assist in the elimination of bilirubin via the liver. For example, in 5 day old hyperbilirubinaemic Gunn pups, hepatic gene expression of CYP1A1/1A2 is increased, with decreases in 7A1 observed, compared to normobilirubinaemic litter-mates. However, unpublished data collected within the Bulmer laboratory demonstrates a 20% increase in gene expression of CYP7A1 in mature female Gunn rats (versus littermate controls). Importantly, CYP7A1 mediates the turnover of cholesterol to bile acids, with increasing expression associated with reduced circulating cholesterol concentrations [23, 31]. It is hypothesised that

UCB accumulation in the blood and tissues of Gunn rats [181], could contribute to decreased circulating cholesterol concentrations observed. Increased CYP7A1 expression, in response to elevated UCB, could facilitate an increased turnover of cholesterol to bile acid, reducing the circulating concentration of cholesterol [31]. The excess bile acids synthesised would then be transported into the blood and bile. This study observed a substantial increase in circulating bile acids, and to a lesser extent, biliary secretion in Gunn rats, supporting this hypothesis.

5.92 Proposal 2: Perturbation of ABCA1 mediated cholesterol efflux reduces circulating cholesterol.

A potential effect of bilirubin on cholesterol efflux represents another possible mechanism to explain hypocholesterolaemia observed in Gunn rats. Wang et al. [169] first observed reduced protein expression and increased degradation of ABCA1 in hyperbilirubinaemic conditions in THP-1 macrophages. ABCA1 is the primary transporter responsible for the efflux of cellular cholesterol to apo-a1 containing lipoproteins (namely HDL)[182, 183], which is a key step in the removal of cholesterol from peripheral tissues and delivery back to the liver. Although this effect has only been observed *in vitro*, the study utilised media supplemented with human/rat plasma from Gunn rats and Gilbert's syndrome individuals, allowing for a closer reflection of physiological hyperbilirubinaemic conditions [169]. It is proposed that the degradation of macrophage ABCA1 by direct interaction with bilirubin, results in decreased HDL formation as observed in this thesis. Such an effect would decrease cholesterol return to the liver via reverse cholesterol transport, acutely decreasing intracellular hepatic cholesterol concentrations. This is expected to activate the sterol regulatory element binding protein (SREBP-2) [32], resulting in upregulation of endogenous cholesterol synthesis, as observed in animals with the lowest HDL concentrations (female Gunn rats). It is, however, acknowledged that hepatic cholesterol content was similar between all groups and this may be explained by

chronically compensated cholesterol synthesis, in response to lifelong hyperbilirubinaemia in the Gunn rats studied. This compensated increase in hepatic cholesterol synthesis could also explain increased biliary lipid excretion and increased bile acid concentrations, because excessive hepatic cholesterol synthesis is often accompanied by increased bile acid synthesis to avoid cholesterol accumulation [30, 32].

Additional studies have demonstrated that agonists of peroxisome proliferator-activated receptors (PPARα) result in increased ABCA1 expression [145]. Interestingly, Stec et. al. [184] observed inhibition of lipid accumulation in bilirubin treated mice, resulting in reduced body fat percentage and reduced total body mass. However, in PPARα knockout models, bilirubin administration did not affect body mass, suggesting PPARα mediates bilirubin's inhibitory effect on lipid accumulation [184]. These data are in agreeance with significant reductions in body weight observed in the female Gunn rats (Figure 4.1). Interestingly, bilirubin and biliverdin are agonistic ligands of PPARα and prevent the accumulation of lipids in peripheral macrophages [169], likely via increased ABCA1 mediated cholesterol efflux. Moreso, activation of PPARα promotes the expression of macrophage SR-BI [185], the primary transporter responsible for the transfer of cholesterol from cells into HDL [186]. These data suggest HDL (apo-a1) specific pathways are primarily targeted by bilirubin, which is also supported indirectly by the current study. The proposed PPARα mediated *increase* in plasma HDL however, does not agree with the results of Wang et al. [169], or by reduced serum HDL concentrations reported in Gunn rats, presented here. These differences, therefore indicate that further studies assessing PPARα activation in Gunn rats are required to confirm whether PPARα is activated in this model, leading to decreased ABCA1 activity.

5.11 Mechanism of Increased Cholesterol Biosynthesis

Increases in endogenous cholesterol synthesis occurs in response to decreased dietary cholesterol intake. With insufficient cholesterol intake and delivery to the liver, SREBP-2 is not inhibited by insig-1, but is instead transported to the nucleus for transcriptional upregulation of HMG-CoA reductase [36]. In instances of reduced circulating or intracellular cholesterol, possibly by Proposal 1/2, the rate of endogenous synthesis will increase as a compensatory mechanism, through the SREBP-2 pathway [32]. This feedback regulation of endogenous synthesis has recently been described as a key mechanism in preventing hypercholesterolaemia [29, 30]. This effect results in overshoot [25, 32, 187] of HMGR upregulation, thereby counteracting the reduced cellular cholesterol concentration. Interestingly, activation of SREBP-2, indirectly causes a decrease in ABCA1 expression, via the CYP7A1/SREBP-2/MiR33A axis [32], thereby highlighting a mechanism that links perturbed cholesterol efflux by ABCA1 (Proposal 2), to increased turnover of cholesterol by CYP7A1 (Proposal 1). Therefore, it is possible that alterations in endogenous cholesterol synthesis are not directly increased by bilirubin, but rather, are increased in response to reductions in hepatic cholesterol, caused either by increased bile acid synthesis (mediated by CYP7A1), or impaired cholesterol efflux from the periphery via degradation of ABCA1.

6.0 Limitations

Liver weight

In this study, the liver weight was not recorded at the time of tissue collection, therefore data including bile flow, hepatic cholesterol content was expressed relative to body weight. These results therefore assume that body weight is a direct reflection of liver size, which, currently has not been proven in Gunn rats. However, Bailey et al. [188] and others [189-191] explored and confirmed the assumption made in previous publications, that liver weight increases in proportion to body weight in Wistar and Sprague Dawley rats. Maerckx et al. [192] has calculated the number of hepatocytes based on a predetermined ratio (5 billion cells/kg body weight).

Blood biochemistry - LDL

The expected concentrations of LDL fell below the accepted detection limits of the analyser used in this study and therefore, these data were not reported. As an additional option, LDL concentrations were calculated using the Friedewald equation [193]. However, quantification of LDL using this equation assumes that the lipid profile of Gunn rats share the same lipid ratios as wild type animals, which was not applicable in the current study. Therefore, the decision was made not to include this data in the final results section. Numerous studies have explored alternative methods of LDL quantification, commonly noting the shortcomings of the original formula, especially in extremes of triglyceride and total cholesterol values [194].

Use of enzymatic assays

Analysis of biliary cholesterol concentrations resulted in low and variable results, suggesting matrix effects were influencing the accuracy of data. It is acknowledged these matrix effects can decrease biliary cholesterol concentrations, below those published in the literature. This suggested that biliary cholesterol output has been underestimated in this study. Fortunately,

additional aliquots of bile have been stored for analysis of sterol content by liquid extraction followed by Gas-Chromatography to eliminate possible matrix effects.

Effects of fasting on bilirubin and cholesterol metabolism

A number of studies [195-197], have outlined changes in bilirubin, and lipid metabolism as a consequence of fasting and could potentially affect tissue lipid content. This study design aimed to reduce variability by keeping the fasting time (6-8 hours) and tissue collection times (~12:00, 14:30, 16:30) consistent. It is unlikely therefore, that fasting and diurnal effects would have dramatically influenced the outcomes of this study.

Endogenous cholesterol synthesis

This analysis of endogenous cholesterol biosynthesis was intended to control for dietary cholesterol intake, total plasma cholesterol concentrations and bile acid/cholesterol excretion. The data currently presented, demonstrates changes in the fractional cholesterol synthesis rate (ie. the proportion of new cholesterol synthesised per unit time, relative to the total cholesterol pool). The data indicate that female Gunn animals have increased fractional cholesterol synthesis, implying that they are synthesising more cholesterol. However, this result may be misleading, because the cholesterol pool itself may be reduced in these animals, and requires determination. This can be calculated through assessment of cholesterol intake (which is negligible (see appendix 1) and output through the faeces (neutral sterol, including bile acid assessment). Although the fractional synthesis rate this is not the most accurate representation of endogenous synthesis, previous studies have reported the same parameter and assumed cholesterol pool sizes remain unchanged [29, 198]. Future analysis of faecal cholesterol and bile acids, from stored samples, will give insight into absolute endogenous synthesis rates between the groups.

7.0 Future Directions

7.0 Future directions

The current study was successful in determining differences in generalised mechanisms of cholesterol metabolism, including increased food intake, increased endogenous cholesterol synthesis, increased biliary cholesterol secretion and decreased circulating cholesterol; all of which are associated with the Gunn phenotype. Furthermore, it was the first to cohesively assess these factors, allowing for analysis of lipid metabolism that assesses the interrelation between these mechanisms. Although this study gives a broad overview concerning the effects of UGT1A1 impairment and/or hyperbilirubinaemia, further analysis, from stored samples, in each of the key stages of cholesterol homeostasis (synthesis, transport and excretion) is required to fully explain the observations seen in the current study.

Further investigation into the expression and function of key enzymes involved in the efflux of cholesterol from the liver and periphery, namely ABCA1, will test the hypothesis outlined in Proposal 2. Similarly, expression analysis of SREBP-2, CYP7A1 and MiR33A will provide insight into the mechanisms regulating bile acid synthesis as discussed in Proposal 1 and confirm the possible down regulation of ABCA1, by activation of SREBP-2, as reported previously [32].

8.0 Conclusion

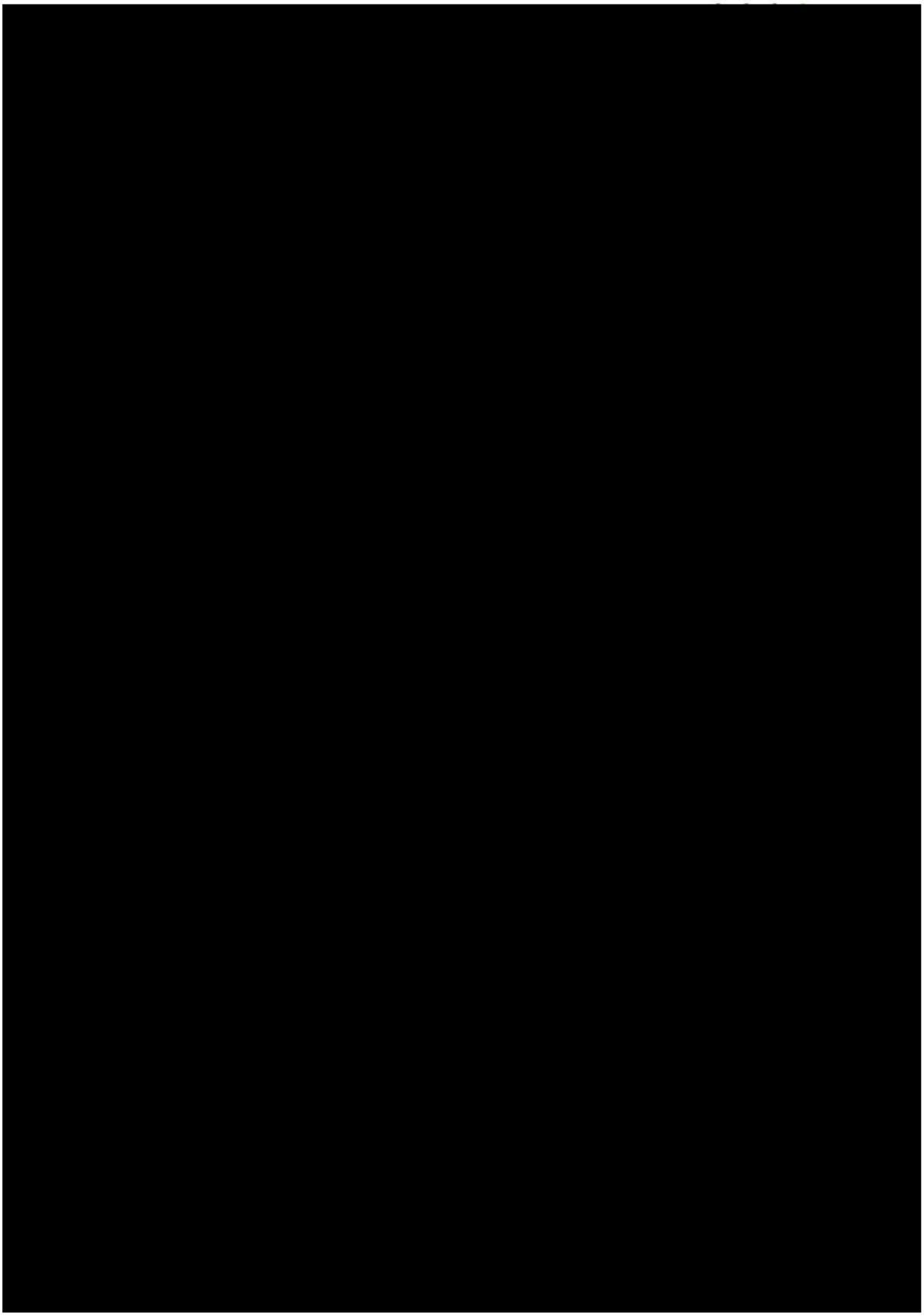
8.0 Conclusion

In summary, this study is the first to assess fractional cholesterol synthesis in Gunn rats, and to compare lipid metabolism in both male versus female and pre-pubescent versus adult rats. Previous literature in the field is limited to assessment of cholesterol sub-fractions and triglyceride content in blood, within hyperbilirubinaemic rodents and humans. However, little understanding regarding the cause of these differences exists. Therefore, this study will assist in understanding the function of the liver (principally endogenous cholesterol synthesis and biliary secretion of bile acids, phospholipids and cholesterol) in mutant hyperbilirubinaemic Gunn rats, and determine whether *de novo* synthesis or excretory processes might be responsible or implicated in the hypocholesterolaemic effect observed in female Gunn animals. The primary hypothesis of this study was that a reduced rate of cholesterol synthesis rate would be observed in Gunn rats, to account for the decreased concentration of circulating cholesterol in these animals. However, this hypothesis was rejected because fractional cholesterol synthesis was increased in female Gunn rats, compared to controls. This finding may reflect a counter-regulatory response, aimed at preserving hepatic cholesterol content in female Gunn animals. The second hypothesis indicated that biliary cholesterol secretion would be increased in Gunn rats. This hypothesis was confirmed, only in female Gunn animals, and suggests greater loss of neutral sterols from the liver, which could lead to depletion of hepatic and circulating cholesterol (if not reabsorbed). Interestingly, the serum concentrations of bile acids were notably increased in Gunn rats, thereby forming a basis of *Proposal 1: Increase in CYP7A1 mediated bile acid synthesis*. Decreased plasma HDL, in conjunction with decreased ABCA1 expression may indicate perturbed export of HDL from the periphery, thereby reducing circulating cholesterol and its delivery back to the liver. This formed the basis of Proposal 2: *Perturbation of ABCA1 mediated Cholesterol Efflux.* The two hypothetical proposals could be combined to explain

increased endogenous cholesterol synthesis in response to acutely reduced hepatic cholesterol (e.g. early in life), thereby increasing biliary cholesterol secretion. Female specific changes in cholesterol metabolism suggests the involvement of sex hormones, which was investigated by comparing serum lipid and bile acid concentrations juvenile Gunn rats, which are theoretically oestrogen naive, to adults. Juvenile female animals did not demonstrate the same phenotype as adult female animals suggesting the involvement of oestrogen in perturbing cholesterol metabolism in Gunn animals. It is hypothesised that circulating oestrogen concentrations would be elevated in adult Gunn rats, due to oestrogen also competing with bilirubin for conjugation by UGT1A1 in a UGT1A1 dysfunctional liver. This hypothesis cannot be adequately addressed in the current study, however comparison of serum oestrogen concentrations in juvenile and adult Gunn rats will provide important information to support future research. Ultimately, this study aimed to provide the first insight into altered cholesterol metabolism in hyperbilirubinaemic animals, the results of which will be built upon using additional tissues collected and stored from this study.

9.0 Appendix

9.1 Appendix 1: Nutritional Information – Chow diet



9.2 Appendix 2 – Tissue Collection Protocol

Study protocol: Bilihealth rat study

We previously observed an increased catabolic shift in the metabolic profile of fasting GS individuals compared to control individuals.

The Bilihealth-rat study shall explore whether catabolism is also induced in Gunn rats and further confirm the expected induction of mitochondrial metabolism by analysing the activity of rate limiting enzymes of mitochondrial energy metabolism.

First NMR and LC-MS analysis will be performed with serum from GUNN and Wistar rats. Then activity of mitochondrial enzymes will be investigated by histochemistry in skeletal muscle, liver and adipose tissue.

Overview of rat studies

Rat study 1:

Juvenile, 3 weeks old Gunn and Wistar rats ~60g body weight (respective to 10 years old human adolescents' prior production of sex hormones) will be fasted for 6 to 7 hours following anaesthesia (Sodium Pentobarb), sacrifice and sample collection.

Heparin Plasma will be sampled from ~40 rats (10 Gunn male, 10 Gunn female, 10 Wistar male, 10 Wistar female), tissue and urine will be collected from at least 28 rats (7 Gunn male, 7 Gunn female, 7 Wistar male, 7 Wistar female). If rats give less than 300µL serum, they cannot be included into the study!

Rat study 2: ([13 C]-acetate intervention)

Gunn and Wistar rats 12-14 weeks, will undergo 19 days of intervention, including [C13] Acetate administration (in water for 5 days), with normal diet. Animals are then undergoing a surgery (fasted) which will last one hour and will then be euthanized.

Heparin Plasma will be sampled from 40 rats (10 Gunn male, 10 Gunn female, 10 Wistar male, 10 Wistar female), tissue, urin, bile and faeces will be collected from 36 rats (9 Gunn male, 9 Gunn female, 9 Wistar male, 9 Wistar female).

Time plan draft

Collection of Tissue

Animals will be fasted for 6 to 7 hours following anaesthesia (Sodium Pentobarb), ~60min surgery (only for study 2), sacrifice and sample collection.

For study 1 and 2 iBAT, iSAT, liver, heparin plasma, gastrocnemius, urine and faeces will be sampled. For study 2 bile will be additionally collected.

All tissues should be collected as quickly as possible. Most importantly, the tissues should be collected within the same time range, order and following the same procedure for each animal

starting with the (1) iBAT (2) iSAT (3) liver (4) heparin plasma (5) gastrocnemius (6) urine (7) faeces. In study 2 bile will be collected during surgery.

Most importantly, identical parts of all tissues are sampled from each animal (babies and adult rats) and all tissues will be frozen immediately.

Timing of the whole sampling procedure will take about ~7 – 10 min.

Adipose tissue

~25mg iBAT (interscapular brown adipose tissue) will be embedded in OTC.

~100mg iBAT will be snap frozen and divided into 2 eppendorfs (min 50mg/Eppendorf). If there is less than 100mg available, tissue will be sampled into 1 eppendorf.

IBAT will be weighted indirect by weighing the animal before and after iBAT removal.

~25mg iSAT (inguinal adipose tissue) will be embedded in OTC.

~300mg iSAT will be snap frozen and divided into 2 eppendorfs (min 100mg/Eppendorf).

Liver

~25mg of the left front lobe will be embedded in OTC.

~600mg of the residual front lobe will be divided into 2 eppendorfs (min 100mg/Eppendorf).

~600mg of the back lobe will be sampled into 1 eppendorf

Most importantly, identical parts of liver and lobe are sampled from each animal.

Heparin plasma:

Minimum: 300µL in 3 aliquots 80µL/100µL/120µL for UCB-HPLC, NMR and UPLC-MS + lipidomics respectively.

If there is between 300 – 600µL plasma it will be added it to the last aliquot.

If there is more than 600µL the last aliquot will be filled up to 500uL and the rest is transferred to a new aliquot.

It is important that the time between blood sampling and centrifugation is comparable between samples. Each blood sample will be centrifuged 5 min after blood collection.

Gastrocnemius

~25mg will be embedded in OTC, a vertical and horizontal cut will be embedded in the same block.

~100mg will be snap frozen and divided into 2 eppendorfs (min 50mg/Eppendorf). If there is less than 100mg available, gastrocnemius will be sampled into 1 eppendorf.

Bile, urine and faeces

~200µL urine will be sampled into 1 eppendorf dissecting the gall bladder.

2 eppendorfs with min 2 pellets (though min 200mg/eppendorf) of faeces respectively will be collected as per 'fresh collection' method upon autopsy from the lower intestine and

immediately frozen in liquid N2. Most importantly, identical stool samples from the lower intestine are sampled from each animal.

~100µL bile will be sampled into 1 eppendorf (only study 2)

Protocol OTC embedding

Length: 0,5cm, width: 0,5cm, no air bubbles, each tissue should be embedded separately,

Orientation when embedding: Again it depends on what you want, have a quick look into a histology book. I usually do cross-sections (the cuts are later done in a ventral-dorsal sequence)

Size of cassette: Doesn't matter unless the person who will be cutting the blocks has a preference.

Manufacturer of OTC: As far as I know only supplied by Sakura Tissue-Tek.

Embedded tissues should be snap-frozen in LN2.

Additional information about the collection of tissue

Rat study 1: (On 25.01.2018: Serum and 1g snap frozen liver of the front lobe has previously been collected from 18 rats)

Rat study 2: (On 25.01.2018: Serum and 1g snap frozen liver of the front lobe has previously been collected from 11 rats. From now on 3 IBATches with 9 rats (3 Gunn male, 3 Gunn female, 3 Wistar male, 3 Wistar female) 1 IBATch every months will be performed.

Reference List

1. Kamphuis, C.B., et al., *Socioeconomic inequalities in cardiovascular mortality and the role of childhood socioeconomic conditions and adulthood risk factors: a prospective cohort study with 17-years of follow up.* BMC Public Health, 2012. **12**: p. 1045.
2. Australian Bureau of Statistics., *3303.0 - Causes of Death*. 2017.
3. Reed, J.R., W.J. Huber, 3rd, and W.L. Backes, *Human heme oxygenase-1 efficiently catabolizes heme in the absence of biliverdin reductase.* Drug Metab Dispos, 2010. **38**(11): p. 2060-6.
4. Anand, S.S., *Quantifying effect of statins on low density lipoprotein cholesterol, ischaemic heart disease, and stroke: systematic review and meta-analysis. Law MR, Wald NJ, Rudnicka AR. BMJ 2003; 326: 1407-408.* Vasc Med, 2003. **8**(4): p. 289-90.
5. Baigent, C., et al., *Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins.* Lancet, 2005. **366**(9493): p. 1267-78.
6. Bruckert, E., et al., *Mild to moderate muscular symptoms with high-dosage statin therapy in hyperlipidemic patients--the PRIMO study.* Cardiovasc Drugs Ther, 2005. **19**(6): p. 403-14.
7. Jick, H., et al., *Statins and the risk of dementia.* Lancet, 2000. **356**(9242): p. 1627-31.
8. Bulmer, A.C., H.J. Verkade, and K.H. Wagner, *Bilirubin and beyond: a review of lipid status in Gilbert's syndrome and its relevance to cardiovascular disease protection.* Prog Lipid Res, 2013. **52**(2): p. 193-205.
9. Tosevska, A., et al., *Longer telomeres in chronic, moderate, unconjugated hyperbilirubinaemia: insights from a human study on Gilbert's Syndrome.* Sci Rep, 2016. **6**: p. 22300.
10. Horsfall, L.J., et al., *Gilbert's syndrome and the risk of death: a population-based cohort study.* J Gastroenterol Hepatol, 2013. **28**(10): p. 1643-7.
11. Welfare, Australian institute of Healthand Welfare., *Trends in Cardiovascular Disease*. 2017, AIIHW: Canberra.
12. Gaziano, T.A., et al., *Growing epidemic of coronary heart disease in low- and middle-income countries.* Curr Probl Cardiol, 2010. **35**(2): p. 72-115.
13. Ademi, Z., et al., *Drug treatment and cost of cardiovascular disease in Australia.* Cardiovasc Ther, 2009. **27**(3): p. 164-72.
14. Gyarfas, I., M. Keltai, and Y. Salim, *[Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries in a case-control study based on the INTERHEART study].* Orv Hetil, 2006. **147**(15): p. 675-86.
15. Castelli, W.P., et al., *Incidence of coronary heart disease and lipoprotein cholesterol levels. The Framingham Study.* JAMA, 1986. **256**(20): p. 2835-8.

16. Anderson, K.M., W.P. Castelli, and D. Levy, *Cholesterol and mortality. 30 years of follow-up from the Framingham study.* JAMA, 1987. **257**(16): p. 2176-80.
17. Nagasawa, S.Y., et al., *Relation between serum total cholesterol level and cardiovascular disease stratified by sex and age group: a pooled analysis of 65 594 individuals from 10 cohort studies in Japan.* J Am Heart Assoc, 2012. **1**(5): p. e001974.
18. Ference, B.A., et al., *Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel.* Eur Heart J, 2017. **38**(32): p. 2459-2472.
19. Bae, J.M., et al., *Low cholesterol is associated with mortality from cardiovascular diseases: a dynamic cohort study in Korean adults.* J Korean Med Sci, 2012. **27**(1): p. 58-63.
20. Jacobs, D., et al., *Report of the Conference on Low Blood Cholesterol: Mortality Associations.* Circulation, 1992. **86**(3): p. 1046-60.
21. Payne, A.H. and D.B. Hales, *Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones.* Endocr Rev, 2004. **25**(6): p. 947-70.
22. Hofmann, A.F. and L.R. Hagey, *Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics.* Cell Mol Life Sci, 2008. **65**(16): p. 2461-83.
23. Pullinger, C.R., et al., *Human cholesterol 7alpha-hydroxylase (CYP7A1) deficiency has a hypercholesterolemic phenotype.* J Clin Invest, 2002. **110**(1): p. 109-17.
24. Siperstein, M.D. and V.M. Fagan, *Feedback control of mevalonate synthesis by dietary cholesterol.* J Biol Chem, 1966. **241**(3): p. 602-9.
25. Jones, P.J., et al., *Dietary cholesterol feeding suppresses human cholesterol synthesis measured by deuterium incorporation and urinary mevalonic acid levels.* Arterioscler Thromb Vasc Biol, 1996. **16**(10): p. 1222-8.
26. Ronda, O.A., et al., *Measurement of Intestinal and Peripheral Cholesterol Fluxes by a Dual-Tracer Balance Method.* Curr Protoc Mouse Biol, 2016. **6**(4): p. 408-434.
27. Honda, A., et al., *Down-regulation of cholesterol biosynthesis in sitosterolemia: diminished activities of acetoacetyl-CoA thiolase, 3-hydroxy-3-methylglutaryl-CoA synthase, reductase, squalene synthase, and 7-dehydrocholesterol delta7-reductase in liver and mononuclear leukocytes.* J Lipid Res, 1998. **39**(1): p. 44-50.
28. Liu, J., et al., *Oxidized low-density lipoprotein increases the proliferation and migration of human coronary artery smooth muscle cells through the upregulation of osteopontin.* Int J Mol Med, 2014. **33**(5): p. 1341-7.
29. Herrema, H., et al., *Bile salt sequestration induces hepatic de novo lipogenesis through farnesoid X receptor- and liver X receptor alpha-controlled metabolic pathways in mice.* Hepatology, 2010. **51**(3): p. 806-16.
30. Schonewille, M., et al., *Statins increase hepatic cholesterol synthesis and stimulate fecal cholesterol elimination in mice.* J Lipid Res, 2016. **57**(8): p. 1455-64.
31. Li, T., et al., *Overexpression of cholesterol 7alpha-hydroxylase promotes hepatic bile acid synthesis and secretion and maintains cholesterol homeostasis.* Hepatology, 2011. **53**(3): p. 996-1006.
32. Li, T., et al., *Regulation of cholesterol and bile acid homeostasis by the cholesterol 7alpha-hydroxylase/steroid response element-binding protein 2/microRNA-33a axis in mice.* Hepatology, 2013. **58**(3): p. 1111-21.
33. Iannelli, F., et al., *Targeting Mevalonate Pathway in Cancer Treatment: Repurposing of Statins.* Recent Pat Anticancer Drug Discov, 2018. **13**(2): p. 184-200.
34. Ference, B.A., et al., *Effect of naturally random allocation to lower low-density lipoprotein cholesterol on the risk of coronary heart disease mediated by*

polymorphisms in NPC1L1, HMGCR, or both: a 2 x 2 factorial Mendelian randomization study. J Am Coll Cardiol, 2015. **65**(15): p. 1552-61.
35. Shepherd, J., *The role of the exogenous pathway in hypercholesterolaemia.* European Heart Journal (supplements), 2001.
36. Horton, J.D., J.L. Goldstein, and M.S. Brown, *SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver.* J Clin Invest, 2002. **109**(9): p. 1125-31.
37. Jia, L., J.L. Betters, and L. Yu, *Niemann-pick C1-like 1 (NPC1L1) protein in intestinal and hepatic cholesterol transport.* Annu Rev Physiol, 2011. **73**: p. 239-59.
38. Van Eck, M., et al., *Scavenger receptor BI and ATP-binding cassette transporter A1 in reverse cholesterol transport and atherosclerosis.* Curr Opin Lipidol, 2005. **16**(3): p. 307-15.
39. Basso, F., et al., *Hepatic ABCG5/G8 overexpression reduces apoB-lipoproteins and atherosclerosis when cholesterol absorption is inhibited.* J Lipid Res, 2007. **48**(1): p. 114-26.
40. Go, G.W. and A. Mani, *Low-density lipoprotein receptor (LDLR) family orchestrates cholesterol homeostasis.* Yale J Biol Med, 2012. **85**(1): p. 19-28.
41. Soutar, A.K. and R.P. Naoumova, *Mechanisms of disease: genetic causes of familial hypercholesterolemia.* Nat Clin Pract Cardiovasc Med, 2007. **4**(4): p. 214-25.
42. Pagler, T.A., et al., *SR-BI-mediated high density lipoprotein (HDL) endocytosis leads to HDL resecretion facilitating cholesterol efflux.* J Biol Chem, 2006. **281**(16): p. 11193-204.
43. Dikkers, A. and U.J. Tietge, *Biliary cholesterol secretion: more than a simple ABC.* World J Gastroenterol, 2010. **16**(47): p. 5936-45.
44. Wiersma, H., et al., *Hepatic SR-BI, not endothelial lipase, expression determines biliary cholesterol secretion in mice.* J Lipid Res, 2009. **50**(8): p. 1571-80.
45. Kozarsky, K.F., et al., *Gene transfer and hepatic overexpression of the HDL receptor SR-BI reduces atherosclerosis in the cholesterol-fed LDL receptor-deficient mouse.* Arterioscler Thromb Vasc Biol, 2000. **20**(3): p. 721-7.
46. Van Eck, M., et al., *Differential effects of scavenger receptor BI deficiency on lipid metabolism in cells of the arterial wall and in the liver.* J Biol Chem, 2003. **278**(26): p. 23699-705.
47. Ji, Y., et al., *Hepatic scavenger receptor BI promotes rapid clearance of high density lipoprotein free cholesterol and its transport into bile.* J Biol Chem, 1999. **274**(47): p. 33398-402.
48. Yu, L.Q., et al., *Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(25): p. 16237-16242.
49. Yu, L., et al., *Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol.* J Clin Invest, 2002. **110**(5): p. 671-80.
50. Temel, R.E., et al., *Hepatic Niemann-Pick C1-like 1 regulates biliary cholesterol concentration and is a target of ezetimibe.* J Clin Invest, 2007. **117**(7): p. 1968-78.
51. Kerzner, B., et al., *Efficacy and safety of ezetimibe coadministered with lovastatin in primary hypercholesterolemia.* Am J Cardiol, 2003. **91**(4): p. 418-24.
52. Oude Elferink, R.P., et al., *Regulation of biliary lipid secretion by mdr2 P-glycoprotein in the mouse.* J Clin Invest, 1995. **95**(1): p. 31-8.
53. Baghdasaryan, A., et al., *Role of hepatic phospholipids in development of liver injury in Mdr2 (Abcb4) knockout mice.* Liver Int, 2008. **28**(7): p. 948-58.

54. Klomp, L.W., et al., *Characterization of mutations in ATP8B1 associated with hereditary cholestasis.* Hepatology, 2004. **40**(1): p. 27-38.
55. Deng, B.C., et al., *Novel ATP8B1 mutation in an adult male with progressive familial intrahepatic cholestasis.* World J Gastroenterol, 2012. **18**(44): p. 6504-9.
56. Groen, A., et al., *Complementary functions of the flippase ATP8B1 and the floppase ABCB4 in maintaining canalicular membrane integrity.* Gastroenterology, 2011. **141**(5): p. 1927-37 e1-4.
57. Henkel, A.S., et al., *Hepatic overexpression of abcb11 promotes hypercholesterolemia and obesity in mice.* Gastroenterology, 2011. **141**(4): p. 1404-11, 1411 e1-2.
58. Paulusma, C.C., et al., *Activity of the bile salt export pump (ABCB11) is critically dependent on canalicular membrane cholesterol content.* J Biol Chem, 2009. **284**(15): p. 9947-54.
59. Constantinides, P., J. Booth, and G. Carlson, *Production of advanced cholesterol atherosclerosis in the rabbit.* Arch Pathol, 1960. **70**: p. 712-24.
60. Rafieian-Kopaei, M., et al., *Atherosclerosis: process, indicators, risk factors and new hopes.* Int J Prev Med, 2014. **5**(8): p. 927-46.
61. Tell, G. and S. Gustincich, *Redox state, oxidative stress, and molecular mechanisms of protective and toxic effects of bilirubin on cells.* Curr Pharm Des, 2009. **15**(25): p. 2908-14.
62. Davies, M.J., *Protein oxidation and peroxidation.* Biochem J, 2016. **473**(7): p. 805-25.
63. Steinberg, D., et al., *Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity.* N Engl J Med, 1989. **320**(14): p. 915-24.
64. Kita, T., et al., *Role of oxidized LDL in atherosclerosis.* Ann N Y Acad Sci, 2001. **947**: p. 199-205; discussion 205-6.
65. Raghow, R., *Statins redux: A re-assessment of how statins lower plasma cholesterol.* World J Diabetes, 2017. **8**(6): p. 230-234.
66. Antonopoulos, A.S., et al., *Statins as anti-inflammatory agents in atherogenesis: molecular mechanisms and lessons from the recent clinical trials.* Curr Pharm Des, 2012. **18**(11): p. 1519-30.
67. Blum, A., *HMG-CoA reductase inhibitors (statins), inflammation, and endothelial progenitor cells-New mechanistic insights of atherosclerosis.* Biofactors, 2014. **40**(3): p. 295-302.
68. Antoniades, C., et al., *Rapid, direct effects of statin treatment on arterial redox state and nitric oxide bioavailability in human atherosclerosis via tetrahydrobiopterin-mediated endothelial nitric oxide synthase coupling.* Circulation, 2011. **124**(3): p. 335-45.
69. Quaschning, T., et al., *Increase in nitric oxide bioavailability improves endothelial function in endothelin-1 transgenic mice.* Nephrol Dial Transplant, 2003. **18**(3): p. 479-83.
70. Jones, P.H., et al., *Comparison of the efficacy and safety of rosuvastatin versus atorvastatin, simvastatin, and pravastatin across doses (STELLAR* Trial).* Am J Cardiol, 2003. **92**(2): p. 152-60.
71. Meor Anuar Shuhaili, M.F.R., et al., *Effects of Different Types of Statins on Lipid Profile: A Perspective on Asians.* Int J Endocrinol Metab, 2017. **15**(2): p. e43319.
72. Hilleman, D.E., S.M. Heineman, and P.A. Foral, *Pharmacoeconomic assessment of HMG-CoA reductase inhibitor therapy: an analysis based on the CURVES study.* Pharmacotherapy, 2000. **20**(7): p. 819-22.

73. Soran, H., R. Dent, and P. Durrington, *Evidence-based goals in LDL-C reduction.* Clin Res Cardiol, 2017. **106**(4): p. 237-248.
74. Neese, R.A., et al., *Measurement of endogenous synthesis of plasma cholesterol in rats and humans using MIDA.* Am J Physiol, 1993. **264**(1 Pt 1): p. E136-47.
75. Sudhop, T., et al., *Changes in cholesterol absorption and cholesterol synthesis caused by ezetimibe and/or simvastatin in men.* J Lipid Res, 2009. **50**(10): p. 2117-23.
76. Jakulj, L., et al., *Transintestinal Cholesterol Transport Is Active in Mice and Humans and Controls Ezetimibe-Induced Fecal Neutral Sterol Excretion.* Cell Metab, 2016. **24**(6): p. 783-794.
77. Hansen, T.W., *Neonatal jaundice and scientific fraud in 1804.* Acta Paediatr, 2002. **91**(10): p. 1135-8.
78. Lee, M., et al., *Spontaneous evolution in bilirubin levels predicts liver-related mortality in patients with alcoholic hepatitis.* PLoS One, 2014. **9**(7): p. e100870.
79. Wusthoff, C.J. and I.M. Loe, *Impact of bilirubin-induced neurologic dysfunction on neurodevelopmental outcomes.* Semin Fetal Neonatal Med, 2015. **20**(1): p. 52-57.
80. Wagner, K.H., et al., *Looking to the horizon: the role of bilirubin in the development and prevention of age-related chronic diseases.* Clin Sci (Lond), 2015. **129**(1): p. 1-25.
81. Wallner, M., et al., *Protection from age-related increase in lipid biomarkers and inflammation contributes to cardiovascular protection in Gilbert's syndrome.* Clin Sci (Lond), 2013. **125**(5): p. 257-64.
82. Sakamoto, S., et al., *Glucuronidation converting methyl 1-(3,4-dimethoxyphenyl)-3-(3-ethylvaleryl)-4-hydroxy-6,7,8-trimethoxy-2-naphthoat e (S-8921) to a potent apical sodium-dependent bile acid transporter inhibitor, resulting in a hypocholesterolemic action.* J Pharmacol Exp Ther, 2007. **322**(2): p. 610-8.
83. Ocadlik, I., S. Hlinstakova, and S. Oravec, *Relationship between unconjugated hyperbilirubinemia and lipoprotein spectrum.* Neuro Endocrinol Lett, 2011. **32**(3): p. 360-4.
84. Vitek, L., et al., *Urinary excretion of oxidative metabolites of bilirubin in subjects with Gilbert syndrome.* J Gastroenterol Hepatol, 2007. **22**(6): p. 841-5.
85. Vitek, L., et al., *Gilbert syndrome and ischemic heart disease: a protective effect of elevated bilirubin levels.* Atherosclerosis, 2002. **160**(2): p. 449-56.
86. Kikuchi, G., T. Yoshida, and M. Noguchi, *Heme oxygenase and heme degradation.* Biochem Biophys Res Commun, 2005. **338**(1): p. 558-67.
87. Sticova, E. and M. Jirsa, *New insights in bilirubin metabolism and their clinical implications.* World J Gastroenterol, 2013. **19**(38): p. 6398-407.
88. Maines, M.D. and N. Panahian, *The heme oxygenase system and cellular defense mechanisms. Do HO-1 and HO-2 have different functions?* Adv Exp Med Biol, 2001. **502**: p. 249-72.
89. Maines, M.D., *New insights into biliverdin reductase functions: linking heme metabolism to cell signaling.* Physiology (Bethesda), 2005. **20**: p. 382-9.
90. Salim, M., B.A. Brown-Kipphut, and M.D. Maines, *Human biliverdin reductase is autophosphorylated, and phosphorylation is required for bilirubin formation.* J Biol Chem, 2001. **276**(14): p. 10929-34.
91. Fevery, J., *Bilirubin in clinical practice: a review.* Liver Int, 2008. **28**(5): p. 592-605.
92. Stocker, R., A.N. Glazer, and B.N. Ames, *Antioxidant activity of albumin-bound bilirubin.* Proc Natl Acad Sci U S A, 1987. **84**(16): p. 5918-22.
93. Cui, Y., et al., *Hepatic uptake of bilirubin and its conjugates by the human organic anion transporter SLC21A6.* J Biol Chem, 2001. **276**(13): p. 9626-30.

94. van der Wegen, P., et al., *Successful treatment of UGT1A1 deficiency in a rat model of Crigler-Najjar disease by intravenous administration of a liver-specific lentiviral vector.* Mol Ther, 2006. **13**(2): p. 374-81.
95. Talafant, E. and J. Tovarek, *Factors affecting the rate of bilirubin conjugation in vitro.* Nature, 1959. **183**(4654): p. 111-2.
96. Monaghan, G., et al., *Genetic variation in bilirubin UDP-glucuronosyltransferase gene promoter and Gilbert's syndrome.* Lancet, 1996. **347**(9001): p. 578-581.
97. Erlinger, S., I.M. Arias, and D. Dhumeaux, *Inherited disorders of bilirubin transport and conjugation: new insights into molecular mechanisms and consequences.* Gastroenterology, 2014. **146**(7): p. 1625-38.
98. Keppler, D., *The roles of MRP2, MRP3, OATP1B1, and OATP1B3 in conjugated hyperbilirubinemia.* Drug Metab Dispos, 2014. **42**(4): p. 561-5.
99. Yoshida, T. and G. Kikuchi, *Purification and properties of heme oxygenase from rat liver microsomes.* J Biol Chem, 1979. **254**(11): p. 4487-91.
100. Muraca, M., J. Fevery, and N. Blanckaert, *Relationships between serum bilirubins and production and conjugation of bilirubin. Studies in Gilbert's syndrome, Crigler-Najjar disease, hemolytic disorders, and rat models.* Gastroenterology, 1987. **92**(2): p. 309-17.
101. Jemnitz, K., et al., *ABCC2/Abcc2: a multispecific transporter with dominant excretory functions.* Drug Metab Rev, 2010. **42**(3): p. 402-36.
102. Koopen, N.R., et al., *Impaired activity of the bile canalicular organic anion transporter (Mrp2/cmoat) is not the main cause of ethinylestradiol-induced cholestasis in the rat.* Hepatology, 1998. **27**(2): p. 537-45.
103. Elias, M.M., A.D. Mottino, and E.J. Ochoa, *Transepithelial fate of bilirubin in the isolated rat kidney.* Biochim Biophys Acta, 1997. **1336**(1): p. 7-14.
104. Bulmer, A.C., et al., *Bile pigment pharmacokinetics and absorption in the rat: therapeutic potential for enteral administration.* Br J Pharmacol, 2011. **164**(7): p. 1857-70.
105. Cuperus, F.J., et al., *Acceleration of the gastrointestinal transit by polyethylene glycol effectively treats unconjugated hyperbilirubinaemia in Gunn rats.* Gut, 2010. **59**(3): p. 373-80.
106. Memon, N., et al., *Inherited disorders of bilirubin clearance.* Pediatr Res, 2016. **79**(3): p. 378-86.
107. Wagner, K.H., et al., *Diagnostic criteria and contributors to Gilbert's syndrome.* Crit Rev Clin Lab Sci, 2018. **55**(2): p. 129-139.
108. Burchell, B. and R. Hume, *Molecular genetic basis of Gilbert's syndrome.* J Gastroenterol Hepatol, 1999. **14**(10): p. 960-6.
109. Maruo, Y., et al., *Two Different UGT1A1 Mutations causing Crigler-Najjar Syndrome types I and II in an Iranian Family.* J Gastrointestin Liver Dis, 2015. **24**(4): p. 523-6.
110. Strassburg, C.P., *Hyperbilirubinemia syndromes (Gilbert-Meulengracht, Crigler-Najjar, Dubin-Johnson, and Rotor syndrome).* Best Pract Res Clin Gastroenterol, 2010. **24**(5): p. 555-71.
111. Jansen, P.L., *Diagnosis and management of Crigler-Najjar syndrome.* Eur J Pediatr, 1999. **158 Suppl 2**: p. S89-94.
112. Polgar, Z., et al., *Gunn Rats as a Surrogate Model for Evaluation of Hepatocyte Transplantation-Based Therapies of Crigler-Najjar Syndrome Type 1.* Methods Mol Biol, 2017. **1506**: p. 131-147.
113. McDonagh, A.F., et al., *Biliary excretion of a stretched bilirubin in UGT1A1-deficient (Gunn) and Mrp2-deficient (TR-) rats.* FEBS Lett, 2001. **506**(3): p. 211-5.

114. Ong, K.L., et al., *The relationship between total bilirubin levels and total mortality in older adults: the United States National Health and Nutrition Examination Survey (NHANES) 1999-2004.* PLoS One, 2014. **9**(4): p. e94479.
115. Schwertner, H.A., W.G. Jackson, and G. Tolan, *Association of low serum concentration of bilirubin with increased risk of coronary artery disease.* Clin Chem, 1994. **40**(1): p. 18-23.
116. Vitek, L., *Bilirubin and atherosclerotic diseases.* Physiol Res, 2017. **66**(Supplementum 1): p. S11-S20.
117. Breimer, L.H., et al., *Serum bilirubin and risk of ischemic heart disease in middle-aged British men.* Clin Chem, 1995. **41**(10): p. 1504-8.
118. Kalkan, G.Y., et al., *Serum bilirubin level and aortic intima-media thickness in patients without clinical manifestation of atherosclerotic cardiovascular disease.* Angiology, 2014. **65**(4): p. 314.
119. Nezu, T., et al., *Carotid Intima-Media Thickness for Atherosclerosis.* J Atheroscler Thromb, 2016. **23**(1): p. 18-31.
120. Kimm, H., et al., *Low serum bilirubin level as an independent predictor of stroke incidence: a prospective study in Korean men and women.* Stroke, 2009. **40**(11): p. 3422-7.
121. Stojanov, M., et al., *Total bilirubin in young men and women: association with risk markers for cardiovascular diseases.* Clin Biochem, 2013. **46**(15): p. 1516-9.
122. Clark, J.F., et al., *Bilirubin oxidation products, oxidative stress, and intracerebral hemorrhage.* Acta Neurochir Suppl, 2008. **105**: p. 7-12.
123. Boon, A.C., et al., *Endogenously elevated bilirubin modulates kidney function and protects from circulating oxidative stress in a rat model of adenine-induced kidney failure.* Sci Rep, 2015. **5**: p. 15482.
124. Kundur, A.R., et al., *Mildly elevated unconjugated bilirubin is associated with reduced platelet activation-related thrombogenesis and inflammation in Gilbert's syndrome.* Platelets, 2017. **28**(8): p. 779-785.
125. Kawamoto, R., et al., *Mildly elevated serum bilirubin levels are negatively associated with carotid atherosclerosis among elderly persons.* PLoS One, 2014. **9**(12): p. e114281.
126. Verkade, H.J., *Inhibition of biliary phospholipid and cholesterol secretion by organic anions affects bile canalicular membrane composition and fluidity.* J Gastroenterol, 2000. **35**(6): p. 481-5.
127. Verkade, H.J., et al., *The uncoupling of biliary lipid from bile acid secretion by organic anions in the rat.* Gastroenterology, 1990. **99**(5): p. 1485-92.
128. Apstein, M.D., *Inhibition of biliary phospholipid and cholesterol secretion by bilirubin in the Sprague-Dawley and Gunn rat.* Gastroenterology, 1984. **87**(3): p. 634-8.
129. Verkade, H.J., et al., *Mechanism of bile acid-induced biliary lipid secretion in the rat: effect of conjugated bilirubin.* Am J Physiol, 1993. **264**(3 Pt 1): p. G462-9.
130. Verkade, H.J., et al., *Mechanism of biliary lipid secretion in the rat: a role for bile acid-independent bile flow?* Hepatology, 1993. **17**(6): p. 1074-80.
131. Kajihara, T., et al., *Effects of bilirubin ditaurate on biliary secretion of proteins and lipids: influence on the hepatic vesicle transport system.* J Gastroenterol Hepatol, 1999. **14**(6): p. 578-82.
132. Stocker, R., et al., *Bilirubin is an antioxidant of possible physiological importance.* Science, 1987. **235**(4792): p. 1043-6.

133. Lapenna, D., et al., *Association of serum bilirubin with oxidant damage of human atherosclerotic plaques and the severity of atherosclerosis.* Clin Exp Med, 2018. **18**(1): p. 119-124.
134. Ozeki, M., et al., *High serum bilirubin is associated with lower prevalence of peripheral arterial disease among cardiac patients.* Clin Chim Acta, 2018. **476**: p. 60-66.
135. Jansen, T. and A. Daiber, *Direct Antioxidant Properties of Bilirubin and Biliverdin. Is there a Role for Biliverdin Reductase?* Front Pharmacol, 2012. **3**: p. 30.
136. Boon, A.C., et al., *Reduced circulating oxidized LDL is associated with hypocholesterolemia and enhanced thiol status in Gilbert syndrome.* Free Radic Biol Med, 2012. **52**(10): p. 2120-7.
137. Wu, T.W., K.P. Fung, and C.C. Yang, *Unconjugated bilirubin inhibits the oxidation of human low density lipoprotein better than Trolox.* Life Sci, 1994. **54**(25): p. P477-81.
138. Ziberna, L., et al., *Bilirubin is an Endogenous Antioxidant in Human Vascular Endothelial Cells.* Sci Rep, 2016. **6**: p. 29240.
139. Bakrania, B., et al., *Chronically elevated bilirubin protects from cardiac reperfusion injury in the male Gunn rat.* Acta Physiol (Oxf), 2017. **220**(4): p. 461-470.
140. Erdogan, D., et al., *Low serum bilirubin levels are independently and inversely related to impaired flow-mediated vasodilation and increased carotid intima-media thickness in both men and women.* Atherosclerosis, 2006. **184**(2): p. 431-7.
141. Rodella, L., et al., *Carbon monoxide and biliverdin prevent endothelial cell sloughing in rats with type I diabetes.* Free Radic Biol Med, 2006. **40**(12): p. 2198-205.
142. Vogel, M.E., et al., *Bilirubin Prevents Atherosclerotic Lesion Formation in Low-Density Lipoprotein Receptor-Deficient Mice by Inhibiting Endothelial VCAM-1 and ICAM-1 Signaling.* J Am Heart Assoc, 2017. **6**(4).
143. Wang, L. and L.E. Bautista, *Serum bilirubin and the risk of hypertension.* Int J Epidemiol, 2015. **44**(1): p. 142-52.
144. Fu, Y.Y., et al., *Hyperbilirubinemia reduces the streptozotocin-induced pancreatic damage through attenuating the oxidative stress in the Gunn rat.* Tohoku J Exp Med, 2010. **222**(4): p. 265-73.
145. Chinetti, G., et al., *PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway.* Nat Med, 2001. **7**(1): p. 53-8.
146. Troughtona, J.A., et al., *Bilirubin and coronary heart disease risk in the Prospective Epidemiological Study of Myocardial Infarction (PRIME).* European Journal of Cardiovascular Prevention and Rehabilitation, 2006. **14**: p. 79-84.
147. Seyed Khoei, N., et al., *Mild hyperbilirubinaemia as an endogenous mitigator of overweight and obesity: Implications for improved metabolic health.* Atherosclerosis, 2018. **269**: p. 306-311.
148. Horsfall, L.J., et al., *Serum bilirubin and risk of respiratory disease and death.* JAMA, 2011. **305**(7): p. 691-7.
149. Zucker, S.D., P.S. Horn, and K.E. Sherman, *Serum bilirubin levels in the U.S. population: gender effect and inverse correlation with colorectal cancer.* Hepatology, 2004. **40**(4): p. 827-35.
150. Belo, L., et al., *Body fat percentage is a major determinant of total bilirubin independently of UGT1A1*28 polymorphism in young obese.* PLoS One, 2014. **9**(6): p. e98467.
151. Zhou, J., T.S. Tracy, and R.P. Remmel, *Correlation between bilirubin glucuronidation and estradiol-3-gluronidation in the presence of model UDP-*

glucuronosyltransferase 1A1 substrates/inhibitors. Drug Metab Dispos, 2011. **39**(2): p. 322-9.
152. Stanford, J.A., et al., *Hyperactivity in the Gunn rat model of neonatal jaundice: age-related attenuation and emergence of gait deficits.* Pediatr Res, 2015. **77**(3): p. 434-9.
153. Yamano, S., et al., *Low total cholesterol and high total bilirubin are associated with prognosis in patients with prolonged sepsis.* J Crit Care, 2016. **31**(1): p. 36-40.
154. Harman, N.L., A.R. Leeds, and B.A. Griffin, *Increased dietary cholesterol does not increase plasma low density lipoprotein when accompanied by an energy-restricted diet and weight loss.* Eur J Nutr, 2008. **47**(6): p. 287-93.
155. Chen, J.H., et al., *A Moderate Low-Carbohydrate Low-Calorie Diet Improves Lipid Profile, Insulin Sensitivity and Adiponectin Expression in Rats.* Nutrients, 2015. **7**(6): p. 4724-38.
156. Ha, Y.C. and P.J. Barter, *Differences in plasma cholesteryl ester transfer activity in sixteen vertebrate species.* Comp Biochem Physiol B, 1982. **71**(2): p. 265-9.
157. Tall, A.R., *CETP inhibitors to increase HDL cholesterol levels.* N Engl J Med, 2007. **356**(13): p. 1364-6.
158. Tsutsumi, K., A. Hagi, and Y. Inoue, *The relationship between plasma high density lipoprotein cholesterol levels and cholesteryl ester transfer protein activity in six species of healthy experimental animals.* Biol Pharm Bull, 2001. **24**(5): p. 579-81.
159. Bulmer, A.C., et al., *Improved resistance to serum oxidation in Gilbert's syndrome: a mechanism for cardiovascular protection.* Atherosclerosis, 2008. **199**(2): p. 390-6.
160. Takikawa, H., et al., *Biliary excretion of bile acid conjugates in a hyperbilirubinemic mutant Sprague-Dawley rat.* Hepatology, 1991. **14**(2): p. 352-60.
161. Dikkers, A., et al., *Hepatic ABCG5/G8 overexpression substantially increases biliary cholesterol secretion but does not impact in vivo macrophage-to-feces RCT.* Atherosclerosis, 2015. **243**(2): p. 402-6.
162. Oude Elferink, R.P., et al., *Uncoupling of biliary phospholipid and cholesterol secretion in mice with reduced expression of mdr2 P-glycoprotein.* J Lipid Res, 1996. **37**(5): p. 1065-75.
163. Apstein, M.D. and S.J. Robins, *Effect of organic anions on biliary lipids in the rat.* Gastroenterology, 1982. **83**(5): p. 1120-6.
164. Verkade, H.J., R.J. Vonk, and F. Kuipers, *New insights into the mechanism of bile acid-induced biliary lipid secretion.* Hepatology, 1995. **21**(4): p. 1174-89.
165. Boyer, J.L., *Bile formation and secretion.* Compr Physiol, 2013. **3**(3): p. 1035-78.
166. Sengupta, P., *The Laboratory Rat: Relating Its Age With Human's.* Int J Prev Med, 2013. **4**(6): p. 624-30.
167. Muraca, M. and J. Fevery, *Influence of sex and sex steroids on bilirubin uridine diphosphate-glucuronosyltransferase activity of rat liver.* Gastroenterology, 1984. **87**(2): p. 308-13.
168. Zhang, Y., et al., *Orphan nuclear receptor oestrogen-related receptor gamma (ERRgamma) plays a key role in hepatic cannabinoid receptor type 1-mediated induction of CYP7A1 gene expression.* Biochem J, 2015. **470**(2): p. 181-93.
169. Wang, D., et al., *Bilirubin Decreases Macrophage Cholesterol Efflux and ATP-Binding Cassette Transporter A1 Protein Expression.* J Am Heart Assoc, 2017. **6**(5).
170. Merola, A.J., R.R. Dill, and A. Arnold, *Effects of estrogens on cholesterol biosynthesis and tissue distribution in rats.* Arch Biochem Biophys, 1968. **123**(2): p. 378-84.
171. Mukherjee, S. and A. Bhose, *Studies on estrogen regulation of cholesterol biosynthesis in rat liver microsomes.* Biochim Biophys Acta, 1968. **164**(2): p. 357-68.

172. Fattah, S., P. Augustijns, and P. Annaert, *Age-dependent activity of the uptake transporters Ntcp and Oatp1b2 in male rat hepatocytes: from birth till adulthood.* Drug Metab Dispos, 2015. **43**(1): p. 1-8.
173. Norlin, M., *Expression of key enzymes in bile acid biosynthesis during development: CYP7B1-mediated activities show tissue-specific differences.* J Lipid Res, 2002. **43**(5): p. 721-31.
174. Smiderle, L., et al., *Evaluation of UGT1A1 and SULT1A1 polymorphisms with lipid levels in women with different hormonal status.* Gynecol Endocrinol, 2011. **27**(1): p. 20-6.
175. Robinson, S.H., C. Yannoni, and S. Nagasawa, *Bilirubin excretion in rats with normal and impaired bilirubin conjugation: effect of phenobarbital.* J Clin Invest, 1971. **50**(12): p. 2606-13.
176. Zhu, L., et al., *Hepatocyte estrogen receptor alpha mediates estrogen action to promote reverse cholesterol transport during Western-type diet feeding.* Mol Metab, 2018. **8**: p. 106-116.
177. Palmisano, B.T., et al., *Sex differences in lipid and lipoprotein metabolism.* Mol Metab, 2018. **15**: p. 45-55.
178. Palmisano, B.T., L. Zhu, and J.M. Stafford, *Role of Estrogens in the Regulation of Liver Lipid Metabolism.* Adv Exp Med Biol, 2017. **1043**: p. 227-256.
179. Kapitulnik, J. and F.J. Gonzalez, *Marked endogenous activation of the CYP1A1 and CYP1A2 genes in the congenitally jaundiced Gunn rat.* Mol Pharmacol, 1993. **43**(5): p. 722-5.
180. Li, Y.Q., et al., *Bilirubin and bile acids may modulate their own metabolism via regulating uridine diphosphate-glucuronosyltransferase expression in the rat.* Journal of Gastroenterology and Hepatology, 2000. **15**(8): p. 865-870.
181. Gazzin, S., et al., *Bilirubin accumulation and Cyp mRNA expression in selected brain regions of jaundiced Gunn rat pups.* Pediatr Res, 2012. **71**(6): p. 653-60.
182. Oram, J.F. and R.M. Lawn, *ABCA1. The gatekeeper for eliminating excess tissue cholesterol.* J Lipid Res, 2001. **42**(8): p. 1173-9.
183. Chawla, A., et al., *A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis.* Mol Cell, 2001. **7**(1): p. 161-71.
184. Stec, D.E., et al., *Bilirubin Binding to PPARalpha Inhibits Lipid Accumulation.* PLoS One, 2016. **11**(4): p. e0153427.
185. Chinetti, G., et al., *CLA-1/SR-BI is expressed in atherosclerotic lesion macrophages and regulated by activators of peroxisome proliferator-activated receptors.* Circulation, 2000. **101**(20): p. 2411-7.
186. Hoekstra, M., *SR-BI as target in atherosclerosis and cardiovascular disease - A comprehensive appraisal of the cellular functions of SR-BI in physiology and disease.* Atherosclerosis, 2017. **258**: p. 153-161.
187. Brown, M.S., P.T. Kovanen, and J.L. Goldstein, *Regulation of plasma cholesterol by lipoprotein receptors.* Science, 1981. **212**(4495): p. 628-35.
188. Bailey, S.A., R.H. Zidell, and R.W. Perry, *Relationships between organ weight and body/brain weight in the rat: what is the best analytical endpoint?* Toxicol Pathol, 2004. **32**(4): p. 448-66.
189. Michael, B., et al., *Evaluation of organ weights for rodent and non-rodent toxicity studies: a review of regulatory guidelines and a survey of current practices.* Toxicol Pathol, 2007. **35**(5): p. 742-50.
190. Nirogi, R., et al., What suits best for organ weight analysis: review of relationship between organ weight and body/Brain weight for rodent toxicity studiesVol. 5. 2014. 1525-1532.

191. Piao, Y., Y. Liu, and X. Xie, *Change trends of organ weight background data in sprague dawley rats at different ages.* J Toxicol Pathol, 2013. **26**(1): p. 29-34.
192. Maerckx, C., et al., *Human liver stem/progenitor cells decrease serum bilirubin in hyperbilirubinemic Gunn rat.* World J Gastroenterol, 2014. **20**(30): p. 10553-63.
193. Martin, S.S., et al., *Friedewald-estimated versus directly measured low-density lipoprotein cholesterol and treatment implications.* J Am Coll Cardiol, 2013. **62**(8): p. 732-9.
194. de Cordova, C.M. and M.M. de Cordova, *A new accurate, simple formula for LDL-cholesterol estimation based on directly measured blood lipids from a large cohort.* Ann Clin Biochem, 2013. **50**(Pt 1): p. 13-9.
195. Griffin, P.M., S.L. Elliott, and K.J. Manton, *Fasting increases serum bilirubin levels in clinically normal, healthy males but not females: a retrospective study from phase I clinical trial participants.* J Clin Pathol, 2014. **67**(6): p. 529-34.
196. Kotal, P., L. Vitek, and J. Fevery, *Fasting-related hyperbilirubinemia in rats: the effect of decreased intestinal motility.* Gastroenterology, 1996. **111**(1): p. 217-23.
197. Meyer, B.H., et al., *The effect of fasting on total serum bilirubin concentrations.* Br J Clin Pharmacol, 1995. **39**(2): p. 169-71.
198. Oosterveer, M.H., et al., *High fat feeding induces hepatic fatty acid elongation in mice.* PLoS One, 2009. **4**(6): p. e6066.