

Department of Endocrinology, Metabolism and Diabetes, Department of Medicine, Karolinska University Hospital, Huddinge, and Center for Nutrition and Toxicology, NOVUM. Karolinska Institute, Stockholm, Sweden

PITUITARY REGULATION OF PLASMA LIPOPROTEIN METABOLISM AND INTESTINAL CHOLESTEROL ABSORPTION

Manuela Matasconi



**Karolinska
Institutet**

Stockholm 2005

Repro Print AB, Stockholm, Sweden
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ISBN 91-7140-571-2

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To Arnaldo and Alberta

(Where there is a will, there is a way)

ABSTRACT

Increased levels of plasma cholesterol are associated with a higher risk to develop atherosclerosis, the leading cause of death in Western societies. The major part of cholesterol in human blood is transported within low-density lipoproteins (LDL-C) that bind to specific cell surface receptors (LDLRs). The liver plays a central role in cholesterol metabolism, regulation of LDLRs, and in the *de novo* synthesis and degradation of cholesterol. The excretion of cholesterol and bile acids from the liver is the major route for elimination of cholesterol from the body. The pituitary exerts important regulatory effects on cholesterol metabolism and the hypophysectomized (Hx) rat constitutes a valuable model to study this regulation. Hx rats show severely altered basal cholesterol metabolism (increased LDL-C, reduced HDL-C, suppressed LDLR and cholesterol 7 α -hydroxylase, CYP7A1), concomitant with a striking loss of resistance to dietary cholesterol. Growth hormone (GH) substitution partly normalizes lipid metabolism of Hx rats but the cause for the lost resistance to dietary cholesterol in Hx rats is still unclear. In this thesis, the role of the pituitary in the regulation of hepatic cholesterol metabolism has been further explored. The main findings are:

- 1) Human GH (hGH) can bind to GH receptors (GHRs) and to prolactin receptors (PRLRs), which are both abundant in the liver. It is demonstrated that the PRLR is not involved in the GH-elicited effects on lipid metabolism of rats; PRL directly administered to Hx rats does not alter the lipid metabolism of Hx rats, and GHs from different species are suitable for research in this field.
- 2) The Hx mouse is shown to be a novel tool for studies on the role of the pituitary in lipid metabolism. The lipid profiles of Hx mice from 3 strains and the LDLR deficient-Hx mouse model have been characterized. The suppression of LDLRs and CYP7A1 in Hx mice cannot fully explain the increased sensitivity to cholesterol feeding following Hx.
- 3) Cholesterol absorption is strongly increased in Hx rats and can be normalized by treatment with ezetimibe.
- 4) Plasma cholesterol increases in old rats and humans. However, intestinal cholesterol absorption is unaltered in old rats, and treatment with GH does not affect it.

In summary, the lactogenic binding of hGH does not affect lipid metabolism of rats. Compared to the Hx rat, the Hx mouse model shows qualitatively similar but less pronounced metabolic aberrations. Cholesterol absorption is under pituitary control in rats, and the increased intestinal absorption of cholesterol in Hx rats may be the cause for the higher sensitivity to dietary cholesterol following Hx of rats.

This study further highlights and extends the role of the pituitary in lipid metabolism *in vivo*, and shows that a most important pituitary control is exerted on intestinal cholesterol absorption.

LIST OF ABBREVIATIONS

ABC	Adenosine triphosphate-binding cassette transporters (ABCA1, -G5, -G8)
ACAT2	Acyl-CoA cholesterol acyl transferase 2
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
ASBT	Apical sodium-dependent bile acid transporter
C4	7 α -hydroxy-4-cholesten-3-one
C7 α OH	Cholesterol 7-alpha hydroxylase
CYP7A1	Cytochrome P450 7A1 enzyme
GH, GHR	Growth hormone, GH receptor
HDL, HDL-C	High-density lipoprotein, HDL-cholesterol
Hx	Hypophysectomy, Hypophysectomized
IGF-1	Insulin-like growth factor-1
LDL, LDL-C LDLR	Low-density lipoprotein, LDL-cholesterol, LDL receptor
MTP	Microsomal triglyceride transfer protein
PRL, PRLR	Prolactin, PRL receptor
NPC1L1	Niemann-Pick C1-Like 1 protein
TG	Triglycerides
VLDL	Very low-density lipoprotein

MAIN REFERENCES

This thesis is based on the following papers, which will be referred to by their Roman numerals

- I. Manuela Matasconi, Bo Angelin, Mats Rudling**
Pituitary control of lipoprotein and bile acid metabolism in male rats: growth hormone effects are not mediated by prolactin.
Am J Physiol Endocrinol Metab. 2004 Jul;287(1):E114-9.

- II. Manuela Matasconi, Paolo Parini, Bo Angelin, Mats Rudling**
Pituitary control of cholesterol metabolism in normal and LDL receptor knock-out mice. Effects of hypophysectomy and growth hormone treatment.
Biochim Biophys Acta - Molecular and Cell Biology of Lipids. 2005 Oct 1;1736(3):221-7

- III. Manuela Matasconi, Cecilia Gälman, Paolo Parini, Bo Angelin, and Mats Rudling**
Loss of resistance to cholesterol feeding in the hypophysectomized rat: Increased cholesterol absorption, which is abolished by ezetimibe treatment.
Manuscript.

- IV. Manuela Matasconi, Cecilia Gälman, Paolo Parini, Bo Angelin, and Mats Rudling**
Intestinal cholesterol absorption is not a major determinant for the age-induced increase in plasma cholesterol: Studies in rats and humans.
Manuscript.

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1 INTRODUCTION

1.1 CHOLESTEROL AND LIPOPROTEIN METABOLISM

Cholesterol is the essential precursor of steroid hormones, bile acids and vitamin D, and is required structurally for cell membranes and myelin sheaths. Plasma cholesterol levels are positively correlated to the development of atherosclerosis, a process initiated by deposition of excess insoluble sterols in the arterial wall [1, 2].

Cholesterol is synthesized from acetate to form a 27 carbon atoms structure organized in cyclohexane rings (A,B,C,D) with methyl groups at C-10 and C-13, a double bond at ring B, and a hydroxyl group (OH) in ring A. Esterification with a long-chain fatty acid (LCFA) at the OH group of cholesterol results in the formation of cholesteryl esters (CE), which are more hydrophobic than cholesterol itself [3].

Body cholesterol is primarily of endogenous origin and its homeostasis involves the movement of cholesterol between peripheral tissues and the liver [4]. The liver regulates the *de novo* synthesis of cholesterol and the excretion of cholesterol into bile (directly or after conversion to bile acids), the secretion of cholesterol into blood as very low-density lipoproteins (VLDL), the modulation of receptor-mediated cholesterol uptake, the formation of CE and the storage of cholesterol. The intestine regulates cholesterol absorption and excretion into feces.

Lipoprotein Metabolism

The transport of lipids in plasma is an essential determinant of normal metabolism. Since lipids are insoluble molecules, they must be “packed” into lipoproteins to be efficiently transported in the blood. Lipoproteins are transported via the exogenous pathway, referring to the metabolism of intestinally derived lipoproteins (dietary lipids), the endogenous pathway, involving the lipoproteins synthesized in the liver, and the reverse cholesterol transport (RCT) referring to lipoproteins carrying lipids from peripheral tissues to the liver (Fig. 1).

Lipoproteins are constantly interchanging particles organized in a hydrophobic core of CE and TG surrounded by a surface amphipatic monolayer of phospholipids, free cholesterol and apolipoproteins (Fig. 1). They are classified into five major groups depending on their electrophoretic mobility (size) and hydrated density[5]. In an order following increasing density and decreasing size, lipoproteins are classified into chylomicrons (CM), very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL), and high-density lipoproteins (HDL). CM and VLDL transport mainly TG, LDL and HDL carry mainly cholesterol. The LDL/HDL (and total cholesterol/HDL) cholesterol ratio is a commonly used predictor for the risk to develop cardiovascular disease [6, 7]. All lipoproteins have surface apolipoproteins that are important for structural stability, for the binding of lipoproteins

to specific receptors, and for the function as co-factors for enzymes. The apolipoprotein Apo-B100 is the structural protein of atherogenic lipoproteins [8].

CMs are produced in the intestine post-prandially, a process mediated by the microsomal TG transfer protein (MTP). CMs enter the lymph and reach the circulation via the thoracic duct. They are composed of ~95% TG and their major apolipoprotein is apoB-48. In peripheral tissues, the TG content of CMs is hydrolyzed by lipoprotein lipase (LPL), and free fatty acids (FFAs) are delivered to muscle and adipose tissue. After exchange of surface lipids and apolipoproteins with HDL, the CM remnants are rapidly cleared by the liver via the apoE-recognizing receptors LDL receptor-related protein (LRP) and LDLR. The half-life of circulating CMs is <15 minutes, and they are thus normally absent in the fasting state.

VLDL are produced in the liver via MTP and secreted into the blood. Their major component is also TG (50-80%). VLDL have a single apoB-100 and acquire apoAs, C, and E in the circulation. The TG content of VLDL is hydrolyzed by LPL via interaction with apoC-II. VLDL remnants are mostly cleared from the circulation via LDLRs. A VLDLR also exists, expressed in heart, skeletal muscle and adipose tissue, which is thought to be involved in the uptake of lipids by peripheral tissues[5]. The half-life of circulating VLDL is 2-3 hours.

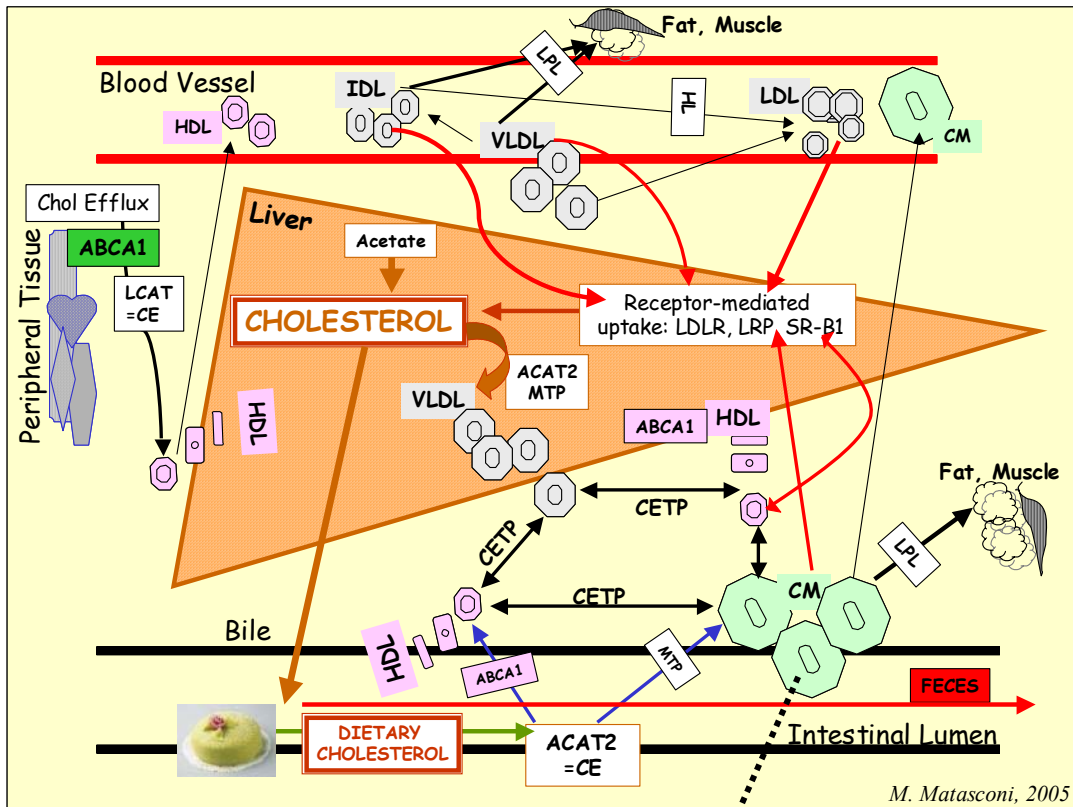
IDL are generated in blood by TG depletion of the VLDL particles. They can be taken up by the liver or, after further hydrolysis of TG via the hepatic lipase (HL), they can be converted into LDL. IDL particles contain multiple copies of apoE and a single apoB-100[9].

LDL are formed in blood following VLDL and IDL delipidation by LPL and HL. LDL particles account for >70% of the cholesterol present in human blood. The only apolipoprotein on the LDL surface is apoB-100. Most LDL-C (70-85%) is removed from the circulation via hepatic LDLRs [1]. A portion of the LDL-C pool is also cleared by a receptor-independent pathway [1]. A structure related to LDL is known as lipoprotein (a), Lp(a). Lp(a) has an additional apolipoprotein, apo(a), bound to its apoB [10], which is thought to be an atherogenic and thrombogenic particle.

HDL are secreted by the liver and intestine as lipid-poor apoA-I that become immediately lipidated by the action of the ATP-binding cassette transporter A1 (ABCA1). In rats and mice, the major fraction of plasma cholesterol is harbored within HDL. Peripheral cells are unable to degrade cholesterol, and mature HDL particles are formed by incorporation of such cholesterol after esterification by lecithin-cholesterol acyltransferase (LCAT). Exchange of CE for TG between HDL and apoB-containing lipoproteins in plasma is catalyzed by the CE transfer protein (CETP). Cholesterol efflux from hepatocytes, enterocytes and extrahepatic tissues has been recently

described as an ABCA1-mediated mechanism [11]. Cholesterol incorporated into HDL particles (HDL-C) is mostly taken up by the liver (85%) by a docking interaction of apoA-I with the scavenger receptor class B type 1 (SR-B1) [12]. HDL promote expulsion of cholesterol from peripheral cells (RCT) and, indirectly, from the body, thus protecting against cardiovascular disease [11].

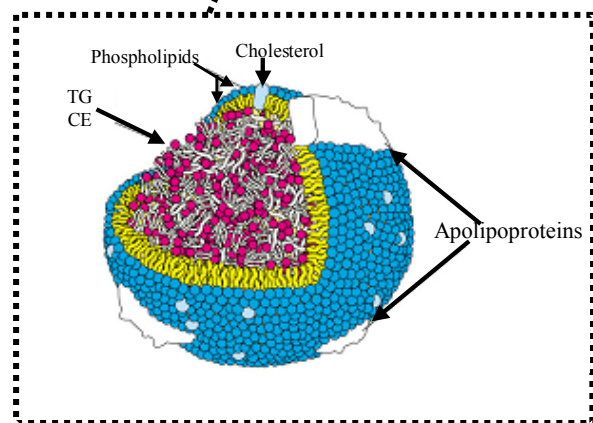
Fig. 1: A schematic overview of lipoproteins lipid transport. Abbreviations are found in the text



The LDL receptor

The concentration of LDL particles in plasma is determined by the balance between LDL synthesis from VLDL/IDL and LDL removal by the hepatic LDLR [9]. The LDLR is synthesized in the endoplasmic reticulum and moves to the cell surface after glycosylation in the Golgi apparatus. On the cell surface, LDLRs concentrate in

clathrin-rich regions called coated pits where endocytosis of the receptor takes place continuously and independently of the presence of a ligand [5, 9, 13, 14]. More than 60% of the LDLRs in the human body are found in the liver [15], where they bind to apoE and apoB-100 containing lipoproteins [9]. Binding to lipoproteins is a calcium-



dependent process [16] that is subject to negative feedback regulation dictated by intracellular cholesterol levels. The sterol regulatory element binding protein 2 (SREBP-2) triggers LDLR (and HMG-CoAR) transcription when the cell is depleted of sterols [17, 18]. When a receptor-lipoprotein complex is formed and internalized, the ligand is degraded in the lysosomal compartment, whereas the receptor is recycled to the surface of the cell several times [14]. Mutations in the LDLR gene cause familial hypercholesterolemia (FH) [9], a disorder resulting in elevated plasma LDL-C, xanthomas, and premature coronary heart disease. The homozygous form is rare (1:1.000.000), the heterozygous form is milder, more frequent (1:350-500), and shows large genetic variability [9, 19, 20]. Heterozygous FH patients usually respond to statins and bile acid binding resins, whereas LDL apheresis is the most effective approach in homozygotes [21]. LDLR-KO mice have been developed to study the role of the LDLR in cholesterol metabolism and may serve as a model to find treatments for FH [22, 23].

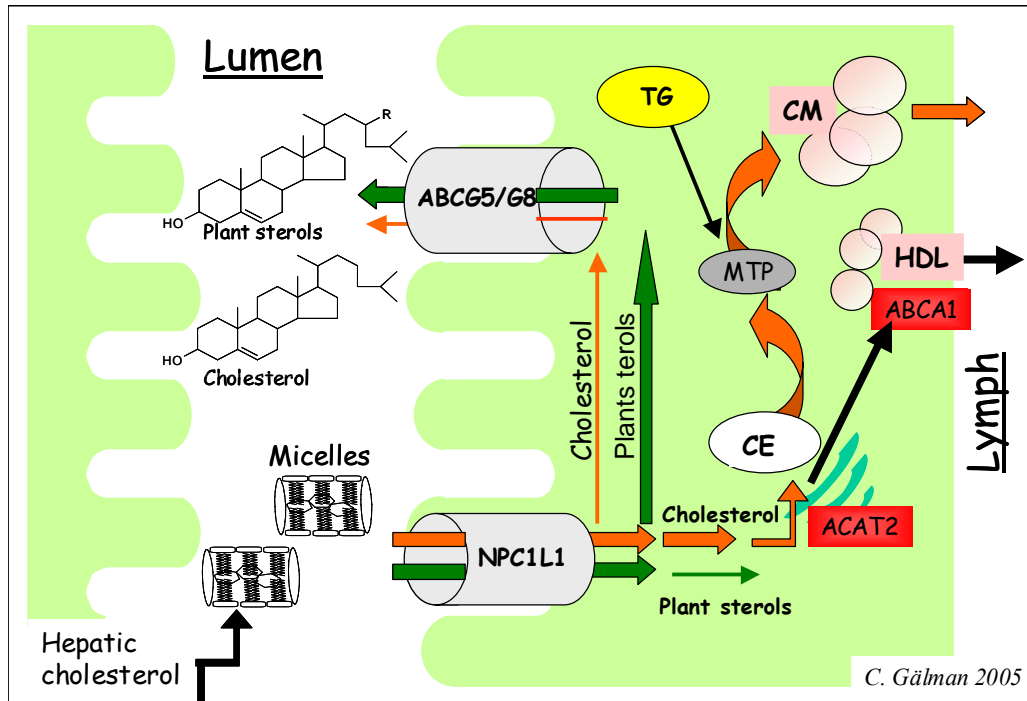
The LDLR expression is under hormonal control both *in vivo* and *in vitro* [24, 25]. Growth hormone (GH) [26, 27], thyroid hormone [28], estrogen [29-32], insulin [33, 34], and glucagon [24] stimulate LDLR, whereas adrenocorticotrophic hormone (ACTH) induces LDLR expression in the adrenals and suppresses it in the liver [35].

1.2 INTESTINAL CHOLESTEROL ABSORPTION

Cholesterol enters the lumen of the small intestine by four main routes: the bile, the diet, the intestinal cells pumping cholesterol back to the lumen, and as cell debris derived from the rapid turnover of intestinal cells. It is mostly absorbed in the duodenum and proximal jejunum [36]. In humans, 30-50% of luminal cholesterol is absorbed and returned to the liver, while the rest is eliminated with feces [37]. Both in humans and rodents, large interindividual and interstrain variations have been reported [36].

Dietary cholesterol is mixed with biliary cholesterol and presented to the brush border membrane of the small intestine in the form of mixed micelles [38, 39]. Cholesterol is transported across the plasma membrane of the enterocyte through a pump recently identified as the Niemann-Pick C1 like 1 protein (NPC1L1) [40]. A fraction of this cholesterol is pumped back into the intestinal lumen by the ATP-binding cassettes hemitransporters ABCG5 and ABCG8, while the remainder moves to the endoplasmic reticulum where it is esterified by the enzyme acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT2) and incorporated into nascent lipoproteins [41, 42](Fig. 2).

Fig.2: Major pathways of micellar cholesterol absorption by the enterocyte. Abbreviations are found in the text.



The **NPC1L1** sterol transporter, also known as NPC3 [43], has 50% aa homology with NPC1, which is involved in intracellular cholesterol trafficking and storage [44]. NPC1L1 is highly expressed in the small intestine of humans and rodents. In humans, it is also found in the liver but its function in this organ is unclear [43, 45]. Also rat liver expresses NPC1L1, in contrast to mouse liver where the expression is low [43-45]. NPC1L1 mice display reduced (~70%) net absorption of cholesterol [43-45]. Absorption of plant sterols is reduced in these mice, indicating that NPC1L1 does not discriminate plant sterols from cholesterol. NPC1L1 has recently been identified as the target for a new class of cholesterol/plant sterol absorption inhibiting drugs [46]; thus NPC1L1-KO mice do not respond to these treatments [44]. Mice depleted of ABCA1 or ACAT2 [47] as well as double KO mice for these genes, all show significantly reduced cholesterol absorption and downregulated NPC1L1, possibly to avoid toxic effects of excess intracellular cholesterol [48]. Many questions concerning NPC1L1 regulation are yet to be answered.

ABCG5 and ABCG8 actively transport cholesterol in the intestine. They are also expressed in the liver where they serve as pumps for the secretion of cholesterol into bile. Unlike ABCA1 and other ABC transporters that encode proteins with 12 transmembrane domains, ABCG5 and ABCG8 each encode a protein with 6 transmembrane domains, therefore a dimerization to form a 12-transmembrane protein complex is required for transport activities [49]. Klett et al. also found evidence of possible functions for ABCG5 and ABCG8 as homodimers or independent of each other [50]. The difference in cholesterol absorption efficiency reported for different

inbred strains of mice was shown to be correlated to the differential intestinal expression of ABCG5/G8 [51]. ABCG5 or ABCG8 overexpression results in a 5-fold increase in biliary cholesterol secretion and a 50% decrease in net cholesterol absorption in mice [52].

ABCs are regulated at the transcriptional level via liver X receptor- α (LXR α) activation [53]. Use of LXR α agonists decreases cholesterol absorption in mice by inducing ABCG5 and ABCG8 [51, 54]. ABCG5/G8 (as ACAT2) mostly regulate cholesterol absorption when dietary cholesterol is significantly raised [37, 47, 51].

Mutations in either hemitransporter cause sitosterolemia, a condition characterized by overabsorption of plant sterols and dietary cholesterol [55-57]. Affected people absorb 15-20% plant sterols instead of the normal 1-3% and have reduced biliary sterol excretion [49, 55]. Plant sterols enter the enterocyte but, being poor substrates for ACAT2, they remain unesterified until they are pumped back to the intestinal lumen by ABCG5/G8 [58]. Plant sterols may be atherogenic compounds that the body efficiently expels as a general defense mechanism [59].

Treatments with bile acid binding resins (cholestyramine) or selective lipase inhibitors (orlistat) reduce cholesterol absorption by interfering with processes other than cholesterol transporters [36, 60]. ACAT2 and partial CETP inhibitors are currently used in clinical trials for the same purpose [61-63].

Ezetimibe

Ezetimibe (EZE) is a potent drug for treatment of sitosterolemia and whose precise mechanisms of action are not completely settled [46, 60, 64]. Sitosterolemic patients do not respond to statin treatments since the accumulation of non-cholesterol sterols in most tissues also disrupts cholesterol homeostasis in the liver, thus resulting in suppression of the *de novo* cholesterol synthesis (the target mechanism of statins). EZE blocks both dietary and biliary cholesterol absorption in the small intestine and is repeatedly delivered to its site of action by enterohepatic circulation [65, 66]. Concomitantly, EZE significantly lowers LDL-C in LDLR-KO mice [67] and in patients with homozygous FH. Enhanced LDLR activity seen in normal situations is therefore a secondary effect of the drug [37]. In combination with statins, EZE further reduces plasma LDL-C in animals and humans [65, 68-72], whereas in combination with bile acid binding resins, it is rapidly excreted and thus less effective [60]. EZE does not interfere with the activity of pancreatic lipolytic enzymes in the intestinal lumen or the bile salt micelle solubilization of cholesterol, nor does it directly affect bile acid metabolism [67, 73].

EZE renders wild-type, SR-B1-KO, and apoE-KO mice resistant to diet-induced hypercholesterolemia [74, 75], whereas it has no effects in mice lacking the NPC1L1 transporter recently identified as its target protein [40, 44, 46]. Interindividual variation in LDL reduction by EZE has been attributed to the existence of different NPC1L1 haplotypes [76]. Whether the NPC1L1 transporter acts in the EZE pathway as a single

unit, complexed to co-factors or whether it serves for intracellular cholesterol trafficking via other mechanisms remains to be elucidated [49].

1.3 THE HYPOPHYSECTOMIZED RAT MODEL

The Pituitary Gland in Lipid Metabolism

The pituitary gland, or hypophysis, is an endocrine organ found at the base of the brain. It is composed of three lobes that can be considered as distinct endocrine organs. The posterior lobe secretes oxytocin and vasopressin. The intermediate lobe is a rudimentary structure composed of agranular cells and thyroid-like follicles. The anterior lobe secretes thyroid stimulating hormone (TSH, thyrotropin), adrenocorticotrophic hormone (ACTH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin (PRL), and growth hormone (GH). PRL acts on the breast, the remaining five are at least in part tropic hormones that stimulate secretion of hormonally active compounds by other endocrine glands or, in the case of GH, the liver or other tissues.

An intact pituitary function is necessary for normal lipoprotein and lipid metabolism [77, 78]. In particular, some anterior pituitary hormones have been shown to regulate lipid metabolism *in vitro* and *in vivo* [79-85]. Most of the *in vivo* data come from studies on the hypophysectomized (Hx) rat model [26-28, 32, 76, 79, 86-90]. As regards lipid metabolism, rats have almost no LDL-C and are resistant to challenge with dietary cholesterol (unaltered total plasma cholesterol and induced LDLR levels [26, 27]). The Hx rat, on the other hand, shows a human-like lipoprotein profile (high LDL-C, low HDL-C) [24, 26] and strongly reduced apoB editing in the liver, so that, as in humans, VLDL particles of Hx rats contain mostly apoB-100 instead of the smaller apoB-48 variant which can be catabolised at a faster rate.

Lipoprotein Profile of the Hypophysectomized Rat

Hx rats fail to grow and reproduce. From a metabolic point of view, hypophysectomy results in many alterations [86]. Total plasma levels of cholesterol may be only slightly increased, whereas TG are reduced to half; However, the small plasma cholesterol increase corresponds to a dramatic change in the lipoprotein profile with a strong increase in LDL-C (5-8 fold) [26, 27]. Concomitantly, cholesterol synthesis, LDLR expression, CYP7A1 activity, fecal bile acid excretion [89], plasma phospholipid concentrations [91], and hepatic FA synthesis (Frick 2002) are reduced, and total liver cholesterol is increased [89]. In contrast to normal rats, Hx rats are highly sensitive to dietary cholesterol [26], and the responses of LDLR to cholesterol feeding [26, 27] or to high doses of estrogen [32] are blunted. Thus hepatic LDLR are markedly reduced in cholesterol-fed Hx rats [26]. Hepatic estrogen receptors are also strongly suppressed in Hx rats and have been shown to be highly dependent on GH levels [27, 89].

Furthermore, the gastrointestinal tract is modified following Hx so that the length of the intestine and the thickness of the mucosa are markedly reduced [92].

1.4 GROWTH HORMONE AND CHOLESTEROL METABOLISM

Physiology of Growth Hormone

Growth hormone (GH) belongs to the Helix Bundle Peptide (HBP) family, a group of structurally related hormones that includes prolactin, placental lactogen, interleukins, colony stimulating factors and macrophage growth factors yielding broad, overlapping physiological functions [93]. GH is produced in the anterior lobe of the pituitary by the somatotrophs whose activity is regulated by the GH-releasing hormone (GHRH) and somatostatin (SS) secreted by the hypothalamus (Fig. 3) [94]. The hypothalamus and some peripheral hormones dictate GH secretion. Insulin-like growth factor-1 (IGF-1) and cortisol in particular exert a negative feedback regulation on GH (Fig. 3). Four different forms of GH exist in humans with the 22kDa form contributing to more than 75% of the circulating GH [95]. Rat GH is transcribed from a single gene and shows 64-67% nucleotide homology with human GH. Bovine and human GH have 75-77% nucleotide homology [96]. A major proportion of GH circulates in blood bound to GH-binding proteins (GH-BPs) that are generated in the liver. Each molecule of hGH binds to two GH-BPs, which derive from cleavage of the extracellular portion of the hepatic GH receptor (GHR) [97]. GH in the free form yields different kinetics and might have different bioactivity [98]. Measure of the level of GH-BPs may serve as a peripheral indicator of the number and activity of the GHRs [94].

There exists a gender differentiated secretion pattern of GH in all mammals [99-101] that leads to gender-dependent differences in lipid metabolism [90, 102-108]. Female rats secrete GH continuously, male rats intermittently, with virtually undetectable GH levels between peaks [101, 109]. These differences occur during sexual maturation, after ~30 days of age [101]. GH secretion peaks in males occur at ~3 hours intervals and range from >200 (highest) to <1 (lowest) ng/mL [109]. No consistent relation between food intake and GH release has been shown [110]. Female, but not male, rats have their highest GH release during the night when the rat is more active [111], in contrast to humans where GH secretion occurs mostly during sleep. GH pulses in humans are found especially during the night and early slow-wave sleep hours [112]. Both women and men secrete GH in pulses (circa 13/d), but women release more GH per day than men, as a result of their higher pulse amplitude and higher baseline levels [112].

Prolactin and Growth Hormone

Prolactin (PRL) is a 23KDa polypeptide essential for reproduction and lactation. PRL shares many functional and structural features with GH. Similarities in the receptors are found in the extracellular binding site. GH and PRL receptors are both activated by sequential dimerization, and the monomeric or dimeric GH-receptor form is strictly dependent on the concentration of the hormone at cell surface. At low GH concentrations, the hormone interacts with one molecule of the receptor named binding site 1. This intermediate is inactive and has to interact with a second molecule of the receptor to form a complex able to trigger intracellular signalling cascades. Human GH presents the unique ability among GHs to bind to the PRL-receptor as well (lactogenic binding) [113-118].

Growth Hormone in Lipid Metabolism

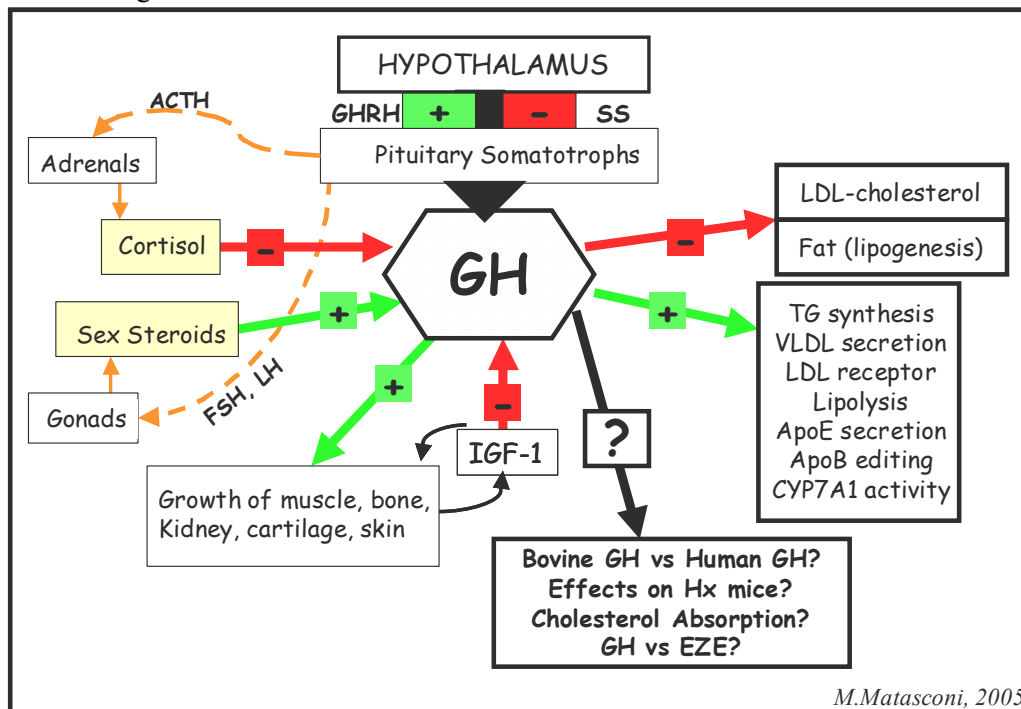
GH has significant effects on lipid metabolism of animals and man [24, 86, 119, 120](Fig. 3). In the liver, GH stimulates the expression of the LDLR [26, 32], the biosynthesis of TG [102], the secretion of VLDL [102] and apoE [105], and the editing of apoB [82]. In adipose tissue, it promotes lipolysis and prevents lipogenesis, thus increasing the availability of FFA for energy expenditure and TG synthesis [24, 121]. In the intestine, GH enhances absorption of calcium and phosphate by increasing intestinal sensitivity to vitamin D. GH stimulates the activity of LPL in heart and skeletal muscle of Hx rats [88]. In plasma, GH reduces LDL-C, and can restore the levels of IGF-1 and of IGF-binding protein 3 (IGFBP-3) in GH-deficient patients [94].

Substitution experiments in Hx rat have shown that GH is essential for normalization of the plasma lipoprotein profile of Hx rats [89, 104, 122, 123]. In addition, in rats the continuous infusion of GH (female secretory pattern ([124, 125]is more effective in normalizing lipoprotein, bile acid (rudling 1997), and FFA metabolism [90] than a pulsatile way of administration (male secretory pattern: sc injections) [90, 102-105, 123, 126]. GH alone reduces plasma LDL in rodents [24, 26, 32] by stimulating LDLR expression [26, 27, 32, 85], apoB editing [122], bile acid synthesis [89], and expression of estrogen receptors [29]. GH therapy in humans has been considered for GH-deficiencies, osteoporosis, and hypopituitary-induced hyperlipidemia [7, 24, 78, 127]. Its use is however still limited due to side effects such as diabetes, hypertension, and fluid retention [94]. GH administration also increases the levels of the atherogenic lipoprotein Lp(a) [103, 119, 127-130] as well as the secretion and plasma concentrations of insulin [103, 131-134]. These effects are not elicited by IGF-1 that yields antagonistic effects with GH for what concerns glucose and FFAs metabolism [103, 127]. IGF-1, on the other hand, cannot stimulate LDLR as GH does [27, 127].

The gender-specific GH secretory pattern, shown to be most important for the regulation of lipid metabolism in rats, may also modulate some parameters in human

lipid metabolism [107]. Thus, in GH deficient humans, subcutaneous injections with GH seem more effective in normalizing lipid profiles than the continuous infusions [128, 135]. GH therapy to GH-deficient adults reduces hepatic cholesterol levels maybe by counteracting dietary-induced synthesis but it does not influence bile acid synthesis, sterol absorption levels, or consistently reduce plasma LDL-C [32, 132, 136-138].

Fig. 3: Overview of the major physiological functions of GH in the body. The “+” sign means stimulation, the “-“ means downregulation, “---“ arrows illustrate pituitary axis, the “?” means unknown mechanisms to be investigated.



Growth Hormone, Dyslipidemia, and Aging

Cholesterol metabolism is profoundly modified during normal aging [139, 140]. Plasma LDL levels increase with age in both humans [141] and rodents [83, 139], contributing to the enhanced incidence of cardiovascular disease with advancing age [141-144]. Studies of 24h GH secretion demonstrated variable reductions (15-70%) in middle-aged and older men and women, with an estimated decrease in GH secretion by 14% for each advancing decade [94, 145]. GH-BPs also decrease after the fifth decade of age, probably reflecting both the reduced levels of circulating GH and hepatic GHRs number. In rats, the age-dependent increase in plasma cholesterol and the reduced bile acid synthesis can be reversed (to levels found in young) following GH administration, indicating that reduced GH secretion during aging is linked to the age-induced metabolic changes [83]. Furthermore, the fact that GH deficiency results in dyslipidemia in humans [120], and that postmenopausal women display higher LDL-C than fertile ones [144], would support this hypothesis also as regards humans. It could be argued that Hx rats may resemble the aging rat/human, especially for what concerns

elevated plasma LDL-C levels, reduced bile acid synthesis, and the responses to dietary cholesterol load.

1.5 BILE ACID METABOLISM

Bile Acid Synthesis

Bile acids are essential for emulsification, digestion and uptake of fat. They are produced in the liver and transported in the bile. Hepatic bile acids are named primary bile acids: cholic acid, CA and chenodeoxycholic acid, CDCA. Before being secreted from the hepatocyte, >98% of the bile acids are conjugated with glycine or taurine to make them more soluble [146]. A portion of primary bile acids is then deconjugated and dehydroxylated in the distal intestine by bacterial enzymes to form secondary bile acids. Being powerful solubilizers of membrane lipids, bile acids are cytotoxic if present at very high concentrations [38, 147]. The rate of bile acid synthesis is tightly regulated to ensure a stable pool size, an adequate fat emulsification in the intestine, and cholesterol homeostasis. It involves at least 17 enzymes [148]; the rate-limiting steps are 7 α -hydroxylation of cholesterol, which is catalyzed by microsomal cholesterol 7 α -hydroxylase (CYP7A1) in the classic (neutral) pathway [146], and 27 α -hydroxylation, catalyzed by mitochondrial sterol 27 α -hydroxylase (CYP27A1), in the acidic (alternative) pathway [149].

Enterohepatic circulation is the name given to the continuous cycle of secretion, absorption and resecretion that distinguishes bile acids. Food intake triggers gallbladder contraction and bile release into the duodenum. More than 95% of bile acids are actively reabsorbed in the distal ileum via the apical sodium-dependent bile acid transporter ASBT (IBAT or human SLC10A), whereas 1-5% continues to the colon to be deconjugated and converted to secondary bile acids [38]. The reabsorbed bile acids are returned to the liver via the portal vein to be resecreted into the bile (enterohepatic circulation). The bile acid pool recirculates 8-10 times per day. Bile acid synthesis in the liver is subject to negative feedback regulation dictated by the flow of bile acids returning to the liver. Interruption of the enterohepatic circulation by bile acid binding resins or ileal resection induce bile acid and cholesterol synthesis, thus leading to upregulation of the LDLR expression and reduction of plasma LDL-C [38, 150].

CYP7A1 activity and expression is strongly suppressed in the Hx model with implications on plasma and liver cholesterol metabolism. CYP7A1 regulation is therefore of importance for this project.

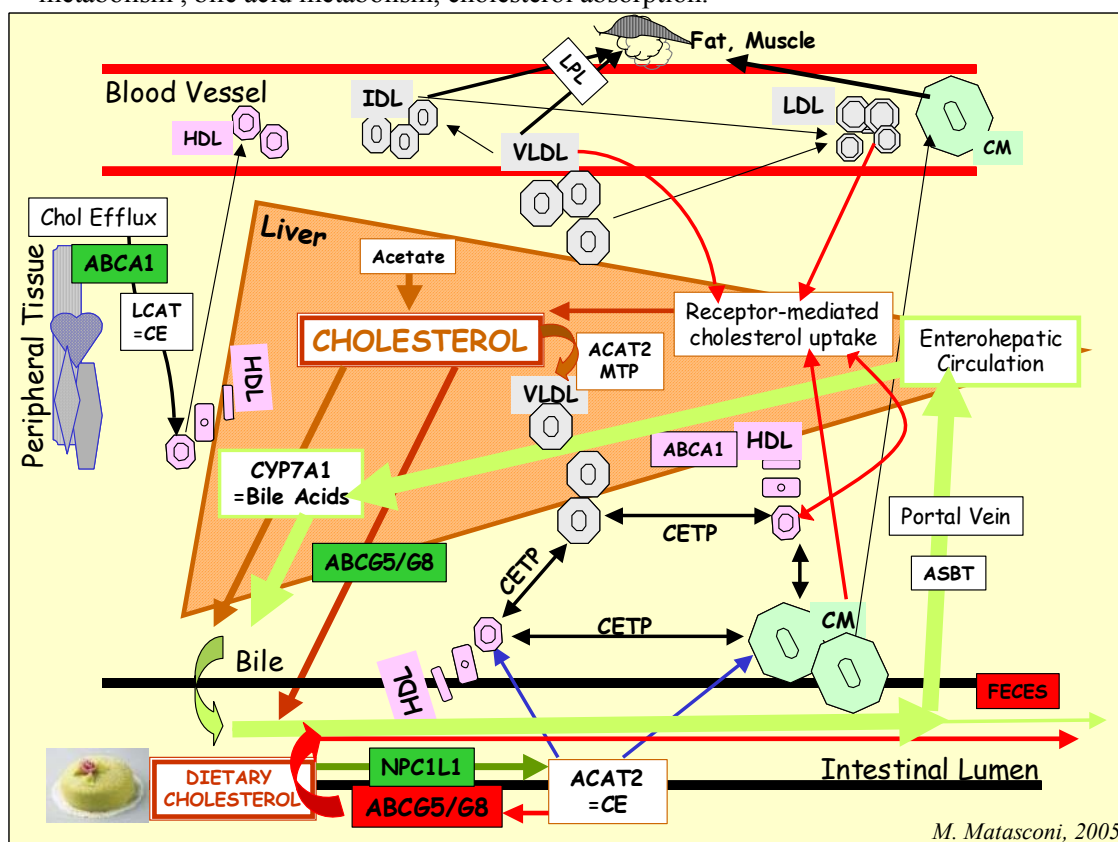
CYP7A1 is transcriptionally regulated. Suppression is triggered by bile acid returning to the liver and binding to the farnesoid X receptor (FXR). When cholesterol accumulates in the body, LXR α activates CYP7A1. When both FXR and LXR α are activated (e.g. by a cholesterol-enriched diet), the stimulatory effect of LXR α on

CYP7A1 overrides the inhibitory action of FXR [151]. Activation of CYP7A1 by LXR α is present in mice and rats but not in humans [152]. The hepatocyte nuclear factors-4 α (HNF-4 α) and -6 (HNF-6) also bind and activate the CYP7A1 promoter [153, 154]. CYP7A1 gene expression is also subject to hormonal regulation. Thyroid hormone, glucocorticoids, and GH stimulate CYP7A1, whereas insulin and glucagon reduce it [28, 89, 146, 155]. A diurnal regulation of CYP7A1 activity has also been demonstrated [146, 156].

CYP7A1-KO mice show high postnatal mortality due to liver failure, lipid malabsorption, and vitamin deficiencies [157], concomitant with reduced bile acid synthesis and bile acid pool size [158, 159]. Plasma lipids are however normal [158], in contrast with the marked hyperlipidemia reported in humans with mutations in CYP7A1 [160].

A recently developed method (C4) allows for measurement of the enzymatic activity of CYP7A1 in serum, thus circumventing the need for liver biopsies (see Experimental Procedures). C4 has also been shown to reflect plasma levels of 7 α -hydroxycholesterol, which in turn mirrors fecal excretion of bile acids [161, 162]. Hence C4 can be regarded as indirect measure of bile acid excretion, in particular in humans where, under physiological conditions, the neutral pathway accounts for ~90% of the total synthesis of bile acids.

Fig. 4 Schematic overview of the pathways described in the Introduction section (lipoprotein metabolism, bile acid metabolism, cholesterol absorption).



M. Matasconi, 2005

2 AIMS

The aim of this project was to improve our understanding of how cholesterol metabolism is regulated by the pituitary. Specifically, the aims were:

1. To evaluate whether pituitary prolactin exerts important effects on lipoprotein and bile acid metabolism in rats, and whether human GH may elicit responses on lipid metabolism that are due to its ability to bind to prolactin receptors in the liver (Paper I).
2. To investigate whether Hx mice can be used to further elucidate the role of the pituitary in lipid metabolism (Paper II).
3. To find the cause for the loss of resistance to dietary cholesterol that occurs in the rat following hypophysectomy (Paper III).
4. To investigate whether the increased plasma lipids found in aging humans and rats could be due to an increase in intestinal cholesterol absorption (Paper IV).

3 EXPERIMENTAL PROCEDURES

The methods used for this project will be summarized below. Details of the procedures are provided in each paper

Animal experiments and tissue collection (paper I-IV)

In the experiments described, either male Sprague Dawley rats, Wistar Hannover rats, or C57BL6J, C3H-Hen, BalbC, LDLR-knockout mice (LDLR-KO) were used. The rats and mice used for papers I-III were hypophysectomized (Hx) via the paraauricular route and intact animals were used as controls. The success of Hx surgery was verified by monitoring failure to gain weight in the operated animals, and by FPLC analysis (see below) of the lipoprotein profile. Bovine and human GH were infused by surgically implanted miniosmotic pumps at the doses indicated; ovine prolactin (oPRL) was injected. Controls were sham operated or injected sc with vehicle. The plasma lipoprotein profile is not altered by a vehicle-filled minipump [83, 163]. The animals were kept under standardized conditions with free access to water and chow. For the rat experiment described in paper IV, 0.5mL blood samples were taken monthly from the tail of 6 young and 6 old rats to monitor eventual age-related changes in plasma lipids. At the end of each experiment, rats were anesthetized with isoflurane and sacrificed by decapitation or cervical dislocation. Blood was thereafter centrifuged, and serum aliquots stored at -80°C ; liver and intestine were excised and snap frozen in liquid nitrogen.

Diets

Standard ground rodent chow was enriched with cholesterol and corn oil for some of the experiments as described. Ezetrol tablets were reduced to powder and mixed with ground chow. Treatments lasted between 7 and 12 days. Where dual-fecal isotope technique was performed (papers III-IV), the rats received a single 0.5mL corn oil intragastric gavage at 9pm with or without isotopes and were then individually housed in metabolic cages for stool collection.

All studies on animals were approved by the Institutional Animal Care and Use Committee.

Human subjects (paper IV)

We assayed the levels of serum plant sterols/cholesterol in a population of 443 human volunteers collected as part of a larger study (Gälman C., unpublished). The study has been approved by the Ethics Committee of the Karolinska Institute, Sweden.

Lipid assays

Total serum cholesterol and triglycerides (papers I-III) were determined using 50µL serum aliquots from all individual animals in a Monarch Automated Analyzer.

Total hepatic cholesterol and triglycerides (Paper III) were measured using commercially available kits after extraction from 50mg liver homogenates according to the method of Folch et al. [164].

Size-fractionation of serum lipoproteins (papers I-IV) was performed by fast performance liquid chromatography (FPLC) system coupled to a system for online separation and detection of cholesterol and TG fractions [81].

Hepatic protein expression

Expression of hepatic LDL-Rs, β -actin, and cholesterol 7 α -hydroxylase (CYP7A1) was assayed on hepatic membrane proteins [32]. LDLR (paper I), was assayed by ligand blot on 6% SDS-PAGE under non-reduced conditions by using ¹²⁵I-labeled rabbit beta-migrating VLDL [32]. Protein expression for β -actin, and cholesterol 7 α -hydroxylase (CYP7A1) [165] was determined by Western blot under reduced conditions using a Criterion™ system. Chemiluminescence substrate was used to visualize the bands on x-ray films.

Hepatic microsomal enzyme activity of CYP7A1 and HMG CoA reductase (paper III)

Microsomes were prepared by differential ultracentrifugation of liver homogenates [166-168]. Microsomal HMG CoA reductase was assayed by determining the conversion of [¹⁴C]HMG CoA to mevalonate [166]. The activity of CYP7A1 was determined as the formation of 7 α -hydroxycholesterol from endogenous microsomal cholesterol using isotope dilution - mass spectrometry [168].

Bile Acid Synthesis (papers I-IV)

7 α -Hydroxy-4-cholesten-3-one (C4) is a serum metabolite that mirrors bile acid synthesis and the enzymatic activity of CYP7A1 [169-173]. Serum samples were diluted with saline and 7 β -hydroxy-4-cholesten-3-one was added as internal standard [174]. The samples were then extracted, eluted, dried under N₂, dissolved in acetonitrile, and separated by HPLC. The wavelength was 241nm.

Quantitative real-time polymerase chain reaction (PCR), (papers III-IV)

Real-time PCR was run by TaqMan™ (paper III) or SYBR Green (papers II-IV) using total RNA extracted with Trizol reagent from snap frozen livers or proximal small intestine. Data were corrected for 18S or GAPDH mRNA. When not available from published papers, primers were designed using Primer Express Software 2.0.

Intestinal cholesterol absorption (papers III-IV)

Fecal dual-isotope method (paper III). Each rat received an intragastric gavage at 9pm containing 5 μ Ci [14 C]-cholesterol and 2 μ Ci [5,6- 3 H]- β -sitostanol in corn oil [159]. Controls received vehicle. Stools were collected and extracted. The percentage of cholesterol absorbed was calculated for each rat from the 14 C / 3 H ratio in feces and in the dosing mixture [159, 175, 176]. In paper IV, all rats were anesthetized with 1:10 hypnorm prior to receiving the ig gavage in order to reduce stress in the aged animals.

Serum plant sterols (papers III-IV). Plant sterols, sitosterol and campesterol, were extracted from 10 μ L (rat) or 20 μ L (human) serum. Samples were analyzed by gas-chromatography-mass spectrometry (GC/MS) using D $_5$ -campesterol/sitosterol as internal standard [177]. The measure of plant sterols in serum mirrors the level of intestinal cholesterol absorption [178]. Experimental models where Western diets are used, are poor indicators of cholesterol absorption since cholesterol competes with plant sterols for uptake in the intestine, thus resulting in lower serum levels of plant sterols despite an increased absorption [36, 179-181].

4 RESULTS

Human and bovine growth hormone elicit similar responses in lipid metabolism of rats (paper I)

Among the pituitary controlled axes (TSH/thyroid hormones, ACTH/cortisol, GH/IGF-1), the putative role of PRL in lipid metabolism is unclear. The fact that PRL treatment of rats increases body weight [182] indicates that PRL may have effects on lipid metabolism. Further, human GH (hGH) can bind to rat PRL receptors [113, 114, 116-118, 136], thereby eliciting PRL effects, in contrast to bovine GH (bGH), which exclusively binds to GHRs [88, 117]. We therefore first investigated whether hGH and bGH had different effects on lipid metabolism of normal and/or Hx rats due to the lactogenic binding of hGH in the liver.

For this purpose, normal Sprague Dawley (SD) rats were infused with bGH or hGH at two different doses. Very similar results were obtained with both hormones on CYP7A1, LDLRs expression, and plasma lipids. We then investigated whether different metabolic responses between bGH and hGH could be found using Hx rats, a deficiency model where GH effects are more pronounced. By treating Hx rats with bGH or hGH, we found that both hormones reduced plasma LDL-C, increased bile acid synthesis, and stimulated LDLR expression to the same extent. Although hGH seemed to have a somewhat stronger response on LDL-C, and bGH had a somewhat better stimulatory effect on the LDLR, these differences were not statistically significant.

We concluded that the important regulatory effects on cholesterol and bile acid metabolism could not be shown to be due to the lactogenic binding attributed to hGH since they were equally well elicited by bGH. Further, even porcine GH (pGH) yielded similar effects on lipid metabolism of normal and Hx rats (Matasconi, unpublished) as shown in Table 1, suggesting that GHs from different species can be employed.

	CHOLESTEROL (mM/L)	TRIGLYCERIDES (mM/L)	C4 (ng/mL serum)
Control Intact	1,9 ± 0,09	1,1 ± 0,13	148 ± 9
Bovine GH dose 1	2,6 ± 0,07	1,4 ± 0,09	268 ± 31
Bovine GH dose 2	2,9 ± 0,06	1,2 ± 0,15	270 ± 26
Human GH dose 1	2,5 ± 0,08	1,0 ± 0,06	282 ± 21
Human GH dose 2	2,4 ± 0,12	1,0 ± 0,06	227 ± 25
Porcine GH dose 1	2,4 ± 0,15	0,9 ± 0,08	217 ± 43
Porcine GH dose 2	2,6 ± 0,06	1,2 ± 0,06	324 ± 21

Table 1: Serum lipids and serum C4 (bile acid synthesis) in normal rats treated with the indicated hormones (dose 1: 1mg/kg/d; dose 2: 3mg/kg/d)

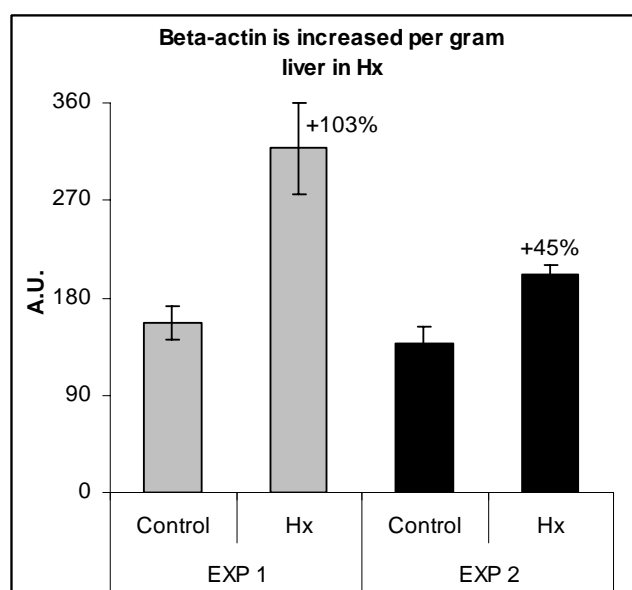
Second, we directly administered ovine prolactin (oPRL) to Hx rats. PRL did not modify the altered lipoprotein profile of Hx rats in contrast to the normalization of

plasma lipoproteins observed with GH treatment. However, PRL caused a significant and dose-dependent increase in body weight in line with previous reports [182]. The latter finding lent further support to the concept that major lactogenic responses are not likely to be involved in the metabolic effects observed following hGH administration to Hx rats.

Hypophysectomy results in reduced levels of total protein and RNA (paper I)

In this work, we raised an important aspect of the Hx animal model, namely the fact that Hx animals show significantly reduced levels of total protein and RNA [183]. Fig. 1 shows the results from Western blots on liver membrane proteins from intact controls and Hx rats from two separate experiments. Beta-actin was strongly increased in Hx rats ($P < 0.001$). This finding is likely due to the fact that there is less total protein and RNA per cell in the liver of Hx animals (and a higher number of cells per gram of tissue). In addition, microarray data published by Flores-Morales et al. [184] showed upregulation of the β -actin gene in liver, heart and kidney from Hx rats. The reduction of total protein and RNA per gram liver in Hx animals can vary from experiment to experiment. Clearly, results from assays that do not correct for this will yield overestimated values for samples from Hx animals.

Fig.1: Beta-actin is strongly increased per gram liver in Hx rats. The graph shows quantified bands (Fuji luminescence image analyzer) from Western blot from two separate experiments on liver membranes from normal (Control) and Hx rats. The gray bars represent experiment 1 (n=5), the black bars experiment 2 (n=4). Error bars represent SEM.



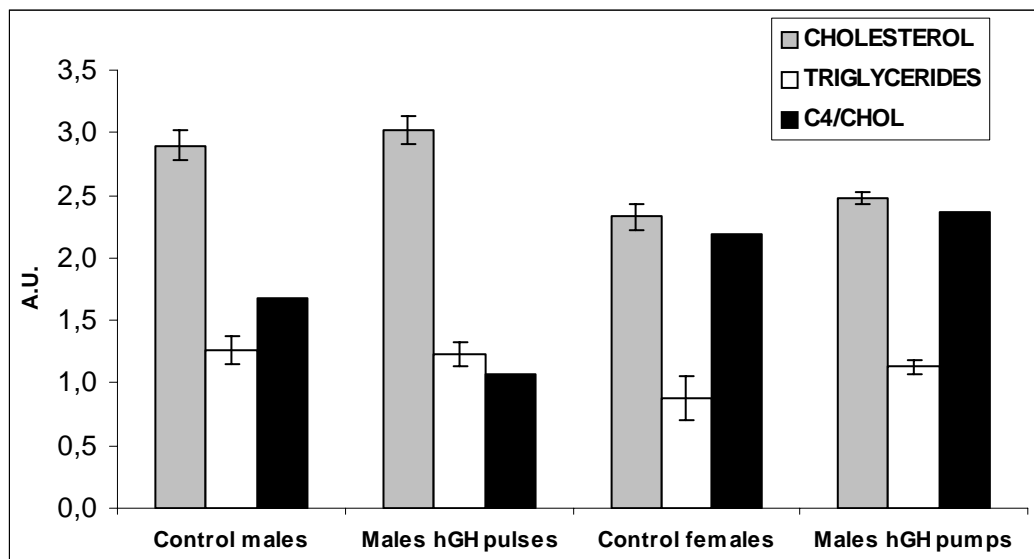
Continuous versus pulsatile way of administration of GH to the mouse

The continuous way of administration of GH mimics the female secretory pattern of normal rats, and is most effective in normalizing serum lipoproteins profiles in Hx rats [102, 105]. We investigated whether also in mice the way of administration of GH determines the magnitude of the effects of GH on cholesterol metabolism. For this purpose, four groups of mice (8-9/group) were employed: one group of females untreated, one of males untreated, one of males treated with hGH delivered by sc

injections (2 x day), and one group of males treated with sc continuous infusion of hGH (to mimic the female secretory pattern). It was indeed found that total cholesterol and serum C4 (bile acid synthesis) were similar in the females as compared to the males treated with continuous GH administration, and in the male controls as compared to the males injected 2 x daily with GH (Fig.2). The regulation depending on the feminine (continuous/pump) and masculine (pulse/injections) secretion patterns of GH previously shown in rats was here demonstrated also in mice.

It can be speculated that gender-related difference in bile acid synthesis in mice (with females having 30-50% higher synthesis than males [52, 185], may be due to the gender-specific secretion pattern of GH. The continuous infusion of GH to males stimulated bile acid synthesis, whereas the 2 injections per day to males rather tended to reduce bile acid synthesis as monitored by serum C4 (Fig. 2). A similar pattern was seen also in the total levels of serum cholesterol: the male controls had similar levels as the males receiving hGH injections, whereas the female controls had similar serum cholesterol levels as the males receiving continuous infusion with hGH (Fig. 2).

Fig.2: masculine (pulse) and feminine (pump) GH administration in mice. For total plasma cholesterol levels, males vs females, $p < 0.01$; males pulses vs males pumps, $p < 0.01$; females vs males pulses, $p < 0.001$; males vs males pumps, $p < 0.05$. The other comparisons were n.s. (Tukey's multiple comparison test).



The hypophysectomized mouse: a new animal model to further study the role of the pituitary in lipid metabolism (paper II)

Here, three main questions were addressed. First, we wanted to characterize the Hx mouse model. Second, we tested the hypothesis whether the established strain-specific susceptibility to dietary-induced hyperlipidemia and atherosclerosis [186, 187] could be due to differences in pituitary control of lipid metabolism. For this purpose, we characterized the plasma lipid profiles of three mouse strains (C57BL6J, C3H-Hen, BalbC) following Hx and upon challenge with a cholesterol/fat diet. Previous work has

shown that the resistance to high fat feeding in these mouse strains is high in C3H-Hen, intermediate in BalbC, and lowest in C57BL6J mice [186, 187]. Third, we aimed to further evaluate the role of the LDLR in the regulation of plasma lipids in the Hx mouse model. Although Hx rats show reduced levels of hepatic LDLR and increased LDL-C that can be almost completely normalized by GH infusion only [26, 32, 89, 188], studies on LDLR-KO mice have indicated that LDLR-independent effects of GH on LDL-C also occur [23]. In order to further investigate the regulation of LDLR in mice, normal, Hx, LDLR-KO and Hx-LDLR-KO mice were employed in one experiment with or without substitution with GH.

We found that: 1) Hx mice may be used in studies on the role of the pituitary on lipid metabolism. Although the changes in cholesterol metabolism observed following Hx of the mouse were qualitatively similar to those occurring in Hx rats, they were clearly quantitatively smaller. 2) The reported particular strain-differences concerning diet-induced hyperlipidemia were actually not found in our experiments, and Hx resulted in similar changes in the three strains investigated (supplement 2, supplementary data paper II). 3) Some effects of GH on plasma lipids are independent of LDLRs since GH reduced serum cholesterol in all lipoproteins in the LDLR-KO and Hx-LDLR-KO mice.

In Hx mice, the basal levels of serum C4 are strongly reduced. In spite of this suppression, however, Hx mice responded to dietary cholesterol with a pronounced increase in serum C4, up to levels seen in normal mice given cholesterol.

This indicates that the reduced resistance to dietary cholesterol also present in Hx mice is not simply due to defective regulation of CYP7A1 or to suppressed LDLR. The primary cause for the loss of resistance to dietary cholesterol may instead reside elsewhere.

Defective regulation of CYP7A1 is not the cause for the increased sensitivity to dietary cholesterol in the Hx rat (paper III)

The unexpected finding of a normal CYP7A1 response to dietary cholesterol in Hx mice (paper II) prompted us to investigate this situation in the thoroughly used Hx rat model where the metabolic changes following Hx are stronger. We found a strong stimulation of C4 (bile acid synthesis) upon cholesterol/fat feeding of Hx rats (paper III). The C4 data were confirmed by measure of CYP7A1 microsomal enzymatic activity, protein expression, and mRNA analysis. This response was surprising since C4 has been repeatedly shown to be strongly suppressed in Hx rats and mice on standard chow [188, 189]. Normal rats respond to dietary cholesterol/fat with small changes in plasma lipids and a modest but consistent increase in C4 levels. This response, present in rats and mice but not in humans, is known to be LXR α -driven [152], and constitutes a most important mechanism for the resistance to dietary cholesterol in these species. LXR-KO mice are in fact very sensitive to cholesterol

feeding [190]. Hepatic LXR α mRNA levels in Hx mice were however unchanged following Hx and upon cholesterol/fat feeding of both normal and Hx mice (Matasconi unpublished).

It was therefore hypothesized that the cause for the increased sensitivity to dietary cholesterol in Hx rats might be due to an increased rate of cholesterol absorption.

Increased cholesterol absorption explains the loss of resistance to dietary cholesterol following hypophysectomy of rats (paper III)

The levels of cholesterol absorbed in Hx rats were assayed by the direct fecal dual-isotope method and indirectly estimated from the levels of plant sterols in serum. The results from the two types of assays were consistent. The fecal dual-isotope assay showed that Hx rats absorb 100% more cholesterol than do the intact animals (32% vs 66%, $p < 0.0001$). These data strongly suggest that the pituitary exerts a very important effect on cholesterol metabolism in the rat by strongly suppressing the absorption of cholesterol in the intestine.

Pituitary-dependent regulation of cholesterol absorption is mediated by the NPC1L1 protein (paper III)

It was hypothesized that the Niemann-Pick C1 Like 1 (NPC1L1) transporter, responsible for the uptake of cholesterol and plant sterols from the lumen into the enterocyte, could mediate the pituitary-dependent increase in cholesterol absorption. We investigated whether the NPC1L1-mediated absorption of cholesterol in the small intestine was induced in Hx rats by using the cholesterol absorption inhibitor ezetimibe (EZE) as tool. Although the mechanism of action for EZE has been much debated, it was recently shown that the NPC1L1 indeed is the target protein for this drug [44, 46]. Overall, EZE reduced serum plant sterols (cholesterol absorption) and plasma cholesterol in Hx rats fed standard chow ($p < 0.001$ for absorption and $p < 0.05$ for LDL-C). Further, in Hx rats fed cholesterol/fat, EZE completely prevented the increase in plasma cholesterol induced by the diets ($p < 0.001$). Moreover, it was found that, although Hx often results in blunted gene and protein expression, the transcript levels for ABCG5/G8, ACAT2 and apoE were all increased in the small intestine of Hx rats, thus indicating that cholesterol uptake was increased, and that LXR α responses were intact in the small intestine of these rats. Given that NPC1L1 resides in an EZE-sensitive pathway responsible for cholesterol absorption, the fact that EZE prevented cholesterol accumulation in serum and liver in cholesterol/fat-fed and, importantly, also in chow-fed Hx rats, suggests that Hx increases the NPC1L1-mediated absorption of cholesterol. The mRNA levels for NPC1L1 were unchanged. However, little is known about the relevance of transcriptional regulation of NPC1L1, and the activity of this protein may reside on its cellular localization [46]. An alternative explanation to our

results could be that the *in vivo* protein activity of ABCG5/G8 is dependent on an intact pituitary. Recent immunoblots on samples from normal and Hx rats did however not reveal any difference as regards the expression of ABCG8 in small intestine (not shown). Nevertheless, if the G5/G8 proteins do not function normally this would lead to an increased absorption of cholesterol despite a “normal“ constitutive expression of the NPC1L1 pump. The latter explanation may be somewhat supported by the finding that feeding Hx rats with 0.4 or 2% dietary cholesterol did not further induce the intestinal mRNA levels for ABCG5 or G8, a response frequently seen in normal animals [37, 51]. However, further extensive experiments using functional assays are needed here.

Overall, study III suggests that the pituitary exerts an important regulation on intestinal cholesterol absorption mediated by the NPC1L1 pump. This could be due to a modulation of the NPC1L1 protein-mediated uptake of cholesterol or to an impaired excretion of cholesterol via ABCG5/G8 via hitherto unknown mechanisms.

The age-induced increase in plasma cholesterol is normalized by GH infusion independently of intestinal cholesterol absorption (paper IV)

Plasma cholesterol increases during aging in rodents and humans [137, 141, 143, 144]. In rats, these metabolic changes with advanced age can be restored by GH infusion [83]. It was here hypothesized that an altered cholesterol absorption could be an important underlying driving force for the age-induced increase in plasma LDL-C.

Lipid profiles were analyzed in response to GH or EZE treatment in old (18mo) and young (6mo) rats. Serum cholesterol levels were monitored prior to the start of the experiment to verify a significant ($p < 0.01$) age-induced increase (not shown).

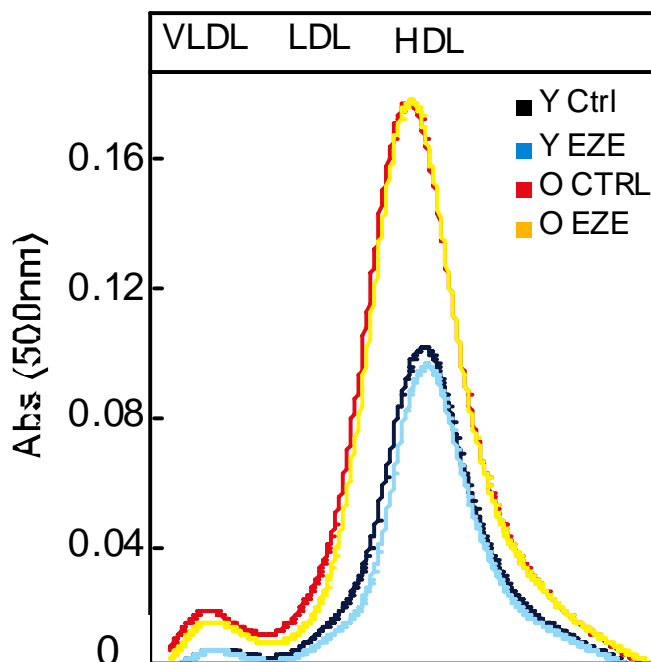
The age-induced increase in serum cholesterol found in the old rats could be reduced by GH infusion in line with previous findings [83]. In young rats, on the contrary, GH administration increased plasma cholesterol as expected [83]. Moreover, CYP7A1 expression was strongly suppressed in old animals and could be restored to normal (young) levels by GH. The gene expression for hepatic LDLR and SR-B1 were also reduced in aged animals and could both be induced by GH treatment.

Cholesterol absorption was not altered by age or by GH treatment in old rats. This suggests that the increased plasma cholesterol during aging is not due to a concomitant increase in intestinal cholesterol absorption. Interestingly, in line with this, EZE had no effect on plasma lipoproteins in young or old rats (FPLC Fig.3), which is also in agreement with previous studies on normal animals [65]. Thus, the FPLC results support the finding of an unaltered cholesterol absorption in this experiment.

In addition, the fact that cholesterol absorption is unchanged in aged rats suggests that that the major determinants of the age-related hyperlipidemia in old rats may be indeed

the reduced expression of hepatic LDLR and SR-B1, the suppressed bile acid synthesis, and the increased cholesterol synthesis.

Fig.3: FPLC lipoprotein profiles in Young and Old Wistar Hannover rats. EZE has no effect on plasma cholesterol profile.



Plasma plant sterol data indicate that cholesterol absorption is reduced with age in humans -despite a clear increase in plasma cholesterol levels (paper IV)

The serum levels of campesterol and sitosterol reflecting the level of cholesterol absorbed in the intestine were measured in a population 443 human volunteers aged 20-80 years. The mean serum LDL-C levels in this population were increased by approximately 40% from age 25 to 65 in both males and females.

The levels of plant sterols showed a progressive and significant reduction with age. This indicates a reduced level of cholesterol absorption with age, supporting the concept (extrapolated from rat data) that changes in intestinal cholesterol absorption do not explain the increase in plasma total and LDL-C occurring with increasing age.

5 GENERAL DISCUSSION

Pituitary hormones have been shown to regulate plasma lipid metabolism ([25, 26, 32, 35, 80, 81, 85, 86, 88, 90, 91, 94, 182] and previous work has shown that GH is particularly important for the regulation of lipid metabolism in rats and humans [23, 24, 26, 32, 78, 83-85, 88, 89, 104, 105, 128, 137, 191]. Until now, these studies have mainly been conducted on hypophysectomized (Hx) rats, whereas the Hx mouse model had not previously been used to study the role of the pituitary in lipoprotein metabolism. An unexplored field is also the potential role of the pituitary in cholesterol absorption. The processes determining intestinal uptake of cholesterol are currently being actively studied and important therapeutic targets for reducing plasma cholesterol levels have been identified [44, 65]. One important aim of the present work was to find the cause for the loss of resistance to dietary cholesterol that occurs following Hx of rats. Cholesterol absorption was investigated in the Hx model as well as in aged rats, displaying age-induced hyperlipidemia [139, 140] –as also occur in aging humans [142].

Human GH can bind both to GH receptors and to prolactin receptors (PRLRs), both abundant in the liver. For this reason, bovine GH (bGH) has commonly been used in lipid research. Moreover, possible direct effects of PRL on lipid metabolism in rats were unclear. From a series of experiments, we demonstrated that the PRLR is not involved in the hGH-elicited effects on lipid metabolism in the rat. PRL directly administered to Hx rats elicited body weight gain but did not alter LDL-C, LDLR expression, or bile acid synthesis. Both bGH and hGH elicited the same responses in normal and Hx rats indicating that the lactogenic binding of hGH did not significantly induce any of the responses observed on lipoprotein metabolism in Hx rats (paper I).

Hx of rats leads to a remarkable loss of resistance to dietary cholesterol along with a strong suppression of bile acid synthesis, and when such animals are challenged with a cholesterol-rich diet plasma cholesterol can increase 5-10 fold [26]. The cause for the loss of resistance to dietary cholesterol following Hx is unclear and might in part involve hepatic LDLR expression and bile acid synthesis that are both reduced in Hx rats.

Pituitary hormones, and particularly GH, regulate plasma lipid metabolism in rats and humans, and can normalize the metabolic changes occurring in Hx rats. GH also increases the expression of LDLR and bile acid synthesis in normal and Hx rats. Although GH is essential for normal enzymatic activity of CYP7A1 and plasma lipoprotein pattern, GH alone cannot restore the reduced fecal excretion of bile acids in Hx rats. Combination with cortisone and T4 is necessary to increase fecal bile acid excretion and GH appears in this situation as a permissive inducer [89]. GH effects on LDLR are direct and not mediated by IGF-1 [27]. However, GH administration to

LDLR-KO mice and Hx-LDLR-KO mice still strongly reduces plasma cholesterol (paper II and [23]), indicating that the LDLR is not a necessary mediator of the effects of GH on plasma cholesterol levels. This reduction may result from reduced levels of hepatic cholesterol and increased bile acid synthesis. Bile acid synthesis is strongly suppressed by Hx and can be stimulated by GH. This effect of GH is elicited also in normal male rats and mice when GH is administrated continuously, mimicking the female secretory pattern of GH, and may be of importance for the gender-specific development of gallstone disease that is more pronounced in male mice than in females. An important prerequisite for gallstone precipitation is a cholesterol supersaturated bile [192].

The Hx rat is a well-characterized model, whereas there is no data on Hx mice. This model has only recently become available much due to the technical difficulty to surgically manipulate small animals. In paper II, we characterized Hx mice from three inbred strains of mice with different degrees of susceptibility to cholesterol feeding. Hx of mice from the three strains resulted in increased VLDL-C and LDL-C. However, Hx of mice caused milder plasma lipids alterations as compared to Hx in rats, and the increase in LDL-C was not as pronounced as in the Hx rat upon cholesterol feeding. Furthermore, the strain differences were not evident in the Hx mouse model, and the responses to dietary cholesterol were similar in normal and Hx mice from the three strains. Thus the pituitary is not a major determinant of the different degree of susceptibility to dietary cholesterol shown for C57BL6J, C3H-Hen and BalbC mice.

Plasma LDL-C was not dramatically increased in Hx mice as it is in the Hx rat, and cholesterol feeding further increased plasma LDL but could never resemble the plasma cholesterol profile found in humans (70% LDL-C) as has been shown for Hx rats fed a Western diet [26]. It can be reasoned that, in normal mice, the normal cholesterol profile consists of a higher LDL peak as compared to the normal rat (VLDL and HDL peaks prominent, almost absent LDL fraction). It can be speculated that this may be due to a much higher bile acid synthesis in the rat [171] and to different GH levels in rat plasma as compared to the mouse. In fact rats grow continuously whereas mice reach a plateau in their growth curve. GH may therefore play an important role in these species differences.

The experiments on Hx mice confirmed that Hx results in loss of resistance to dietary cholesterol. An important finding from paper II was that Hx mice showed strongly increased levels of CYP7A1 when fed a Western diet (2% cholesterol/10%corn oil). This finding was surprising in relation to the previous findings of a strongly reduced expression of CYP7A1 in Hx rats. We do not understand the mechanisms for the suppression of CYP7A1 following Hx. The same regulation was found in the rat study (III) and prompted us to measure the cholesterol absorption in the rat model to

further seek for the cause(s) for the loss of resistance to dietary cholesterol following Hx.

We found that cholesterol absorption is strongly increased in Hx animals and that the cholesterol absorption inhibitor ezetimibe (EZE) could reduce both absorption and serum cholesterol in Hx rats. This finding had three important implications. First, the reduction in plasma LDL-C in chow-fed Hx rats by EZE strongly suggests that the hyperlipidemia in Hx is likely to be caused by an increased intestinal absorption of cholesterol. Second, EZE target-proteins seem not to be modified by Hx since EZE reduces absorption in chow-fed and cholesterol-fed Hx animals (paper III). Third, EZE did not affect plasma lipoprotein profile in young chow-fed animals nor in old chow-fed rats (paper IV). In these animals, absorption was unchanged with age but was reduced by 50% by treatment with EZE. As concerns the old animals, plasma lipids were altered and bile acid synthesis reduced despite the unaltered cholesterol absorption as compared to the younger rats. It would be interesting to study the effects of low doses GH in combination with EZE to reduce plasma lipids from the plasma/liver and intestine/liver compartments respectively.

With the present study, it was further highlighted that the pituitary gland exerts an important role on plasma lipid metabolism and that it also controls the absorption of cholesterol by the intestine. This novel aspect needs further investigation; a better understanding of the mechanisms for the pituitary regulation of cholesterol absorption appears now a necessary and appealing task.

6 CONCLUSIONS

1. The effects of human GH on lipoprotein metabolism in the rat are GH-specific in that they are not mediated by the lactogenic properties of human GH. Pituitary prolactin could not be shown to elicit any of the GH-induced effects on lipid metabolism.
2. The Hx mouse may be employed as model for studies on the role of the pituitary in lipid metabolism. One major advantage of such an approach is the possibility to use genetically modified Hx mice in the future.
3. CYP7A1 is not defective in cholesterol-fed Hx animals, and CYP7A1 and LDLR are not the sole structures responsible for the increased sensitivity to dietary cholesterol in this model. Cholesterol absorption is markedly increased in Hx rats suggesting a central role of the pituitary in the regulation of cholesterol absorption. The induced cholesterol absorption in Hx rats is mediated by the NPC1L1 protein. Since ezetimibe reduces cholesterol absorption also in Hx rats, absorption blockade may be beneficial for patients with hypopituitarism-associated dyslipidemias.
4. The age-dependent increase in plasma lipids occurring in old rats and humans is not linked to increased cholesterol absorption. GH has no effects on cholesterol absorption of aging rats despite its normalizing effects on plasma LDL-C.

7 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Important considerations emerge from the present work that may be useful for future studies.

1) Cholesterol absorption is increased in the Hx model. A substitution experiment with GH, L-thyroxine (T4) and cortisone, alone or in different combinations would identify the hormone(s) responsible for the increased cholesterol absorption in this animal model. The experiments on old rats and a study on GH-deficient humans conducted by Leonsson et al. [137] suggest that hGH has no major effect on cholesterol absorption. Whether this is different in rodents remains to explore.

2) Moreover, it would be of interest to investigate whether low doses of GH might have additive effects on lipid metabolism if used in combination with EZE.

3) Since EZE efficiently reduces cholesterol absorption and plasma lipids in Hx rats, it would be interesting to investigate further the regulation of EZE targets in this animal model. Repa et al. have excluded that the ABCA1/G5/G8 are involved in the EZE-pathways, at least in mice [193], and stated that NPC1L1 is probably not much regulated transcriptionally (Repa personal communication). Since NPC1L1 is definitely involved in the EZE pathways [46], it should be useful to measure NPC1L1 protein expression in our models. Beside the NPC1L1 protein, another candidate target of EZE has been indicated by Smart et al [194], namely the Annexin2-caveolin1 heterocomplex which is important for active absorption and is disrupted by EZE.

4) Most of the data presented in papers III-IV consist of RT-PCR mRNA data and would need further support by analysis of protein expression for the same structures. This is essential for a better understanding of the regulation of the cholesterol transporters.

5) Other intestinal structures may be modified upon hypophysectomy and need further attention. Among these, the heterodimeric organic solute transporter ($\text{ost}\alpha\text{-ost}\beta$) necessary for the ASBT-mediated reuptake of bile acids (sodium-dependent bile acid transporter, human SLC10A2) [195] and mucine-1 (Muc-1) located at the intestinal diffusion barrier shown to be very important for normal sterol uptake [196]. In Muc-1 deficient mice cholesterol absorption is reduced by 50% [36], the status of the intestinal mucous coat is not fully characterized in the Hx model.

6) Hx rats have increased cholesterol absorption but unchanged NPC1L1 mRNA levels. Alterations might occur in the distal ileum, for instance at the level of

ASBT or even FGF-15. ASBT activity may influence cholesterol absorption by modulating bile acid pool size in the intestinal lumen. ASBT-deficient SLC10A2 mice and pharmacological inhibition of ASBT result in reduced plasma cholesterol but only a mild reduction in cholesterol absorption [49]. We hypothesized, however, that this mechanism may be impaired following Hx and possibly improved by hormonal substitution(s).

These observations constitute the basis for our future studies on the role of pituitary hormones in the regulation of plasma lipoproteins and intestinal cholesterol absorption. GH effects on cholesterol absorption will be evaluated. A full substitution experiment will be run to evaluate the role of each hormone (and each combination) on cholesterol absorption. Concomitantly, we will focus on optimising Western blot analyses to determine whether the intestinal cholesterol transporters are subject to transcriptional or post-transcriptional regulations.

8 ACKNOWLEDGEMENTS

I WOULD LIKE TO EXPRESS MY SINCERE GRATITUDE TO ALL THE PEOPLE WHO HAVE CONTRIBUTED, DIRECTLY OR INDIRECTLY, TO THE COMPLETION OF THIS WORK. IN PARTICULAR, I WISH TO THANK

Professor Mats Rudling, my supervisor, for guidance, dedication, encouragement, and great advises. And thanks for answering the phone on Friday evenings and weekends to cope calmly with my crisis situations!

Professor Bo Angelin, my co-supervisor, for invaluable support and for being always positive, no matter what results I get!

Dr Paolo Parini, also co-supervisor, for support in life and work, for the good chats about anything, and for taking care of me on my first visit to Stockholm.

Professor Jan Bolinder and Professor Jan Palmblad, the present and former Head of the Department of Medicine, for providing an excellent working environment.

TACK everybody at Metabollab for the “God Mondag, God Tisdag, God Onsdag, God Torsdag, God Fredag...” x 4.5 years. I had great time learning the job and (almost) the Swedish language from you! Thanks for your patience and friendship Lilian Larsson, Lisbet Benthin, and Ingela Arvidsson. Thanks Lisa-Mari Nilsson and Dr. Ewa Ellis for the good time in and outside the lab. Thanks Catharina, Katarina, Sabine, Pia, Janne, Britt-Marie, Wiveka, Ewa for making the kitchen (and Solvalla) a nice place to be! Thanks Lena and Åse for being always helpful and smiley.

TACK everybody at Novum: Inger, Inger, Inger and all the other “smiley hard workers” crowding the corridors. In particular STOR TACK to Camilla Pramfalk, Thomas Lundåsen, Lisen Johansson, Ylva Bonde, it has been great to share office, lab benches, and basically most of my time in Sweden with you! And thanks to Dr. Cecilia Gälman, for being friend first, “guide of Sweden” then, collaborator in the end. I could not have been luckier!

TACK TACK Jenny and Per-Erik (and Lillan) for sharing the flat with me and introducing me to the delicacies from Northern and Southern Sweden, for the good nights out and the dark days in, for the nice chats, for the patience with foreigners on the phone and the loud guests. Thanks for being “my Swedish family”

A VERY SPECIAL THANKS to Margherita, Milena, Mauro, Martina, Jordan, Marco, Danilo, Stefano, Umberto, Salvatore, Fabio, Roberta, Lella, Marco, Sonia, Alessia, Valeria, Matteo, Ylenia, Nonna Rina, Peppe T., Flavio, Luisa, Antonella, Gigi, Rocco, Angela T., Angela P., Pina, Claudio, Peppe F., Lisa, Mimma, Speranza & Co (and many others) for being there...when I wasn't.

THANKS also to all the friends I have met and lived with everywhere, who live and come from everywhere...for always being ready to meet up for a cheers, a prost, a skål, and amusing chats

M. Matasconi

about life, love, careers, and friendship: Nanyi, Keiko, Amour, Marcel, Marcelline, Maddalena, Umberto, Bettina, Sally, Glenn, Malin, Adela, Angel, Herko, Cristian, Hans, Linda, Jimbo, ...

And GRAZIE to the friends who are “stationary” in the Sacrofano/Rome area: Francesca, Linda, Cristina, Genni, Fabiola, Mara, Serena, Fabrizio, Ermanno, Luigi, Stefania, Donatella, Rita...for being there for me anytime I have 2 minutes to meet up, or I need a lift to/from the airport, and for showing me I am still part of the gang!

A BIG THANKS and a BIG HUG to my sister Ba, who is my complete opposite and with whom I always have so much fun!

THANKS to my parents Arnaldo and Alberta, for waiting patiently.

This work was supported by grants from the Swedish Research Council, the Swedish Foundation for Strategic Research, the Grönberg foundation, the Swedish Heart-Lung Foundation, the "Förenade Liv" Mutual Group Life Insurance Co., the foundation of Old Female Servants, and the Karolinska Institute.

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