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Investigations on extra- and intracellular retinol-binding proteins

Dissertation zur Erlangung des akademischen Grades des

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2. Gutachter: Dr. Silke Vogel (Assistant Professor)
3. Gutachter: Prof. Dr. Walter Zidek

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2. Effect of renal replacement therapy on retinol-binding protein 4 isoforms
   Frey SK, Nagl B, Henze A, Raila J, Tepel M, Schweigert FJ, Zidek W. Clin Chim Acta. 2009 Mar;401(1-2):46-50.

3. Evidence that kidney function but not type 2 diabetes mellitus determines retinol-binding protein 4 (RBP4) serum levels.
   Henze A, Frey SK, Raila J, Tepel M, Scholze A, Pfeiffer AF, Weickert MO, Spranger J, Schweigert FJ. Diabetes. 2008 Dec;57(12):3323-6.

4. Isoforms of retinol-binding protein 4 (RBP4) are increased in chronic diseases of the kidney but not of the liver.
   Frey SK, Nagl B, Henze A, Raila J, Schlosser B, Berg T, Tepel M, Zidek W, Weickert MO, Pfeiffer AF, Schweigert FJ. Lipids Health Dis. 2008 Aug 27;7(1):29.

The second part of this work investigating cellular retinol-binding proteins in mice has been carried out with Dr. Silke Vogel at the Department of Medicine, Columbia University, New York City and has been supported by the German Academic Exchange Servie (DAAD).

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Abbreviations

ARAT – Acyl-CoA:retinol acyltransferase

BMI – Body mass index

CRBP-I – Cellular retinol-binding protein type I

CRBP-I KO – CRBP-I knock-out

CRBP-II – Cellular retinol-binding protein II

CRBP-III – Cellular retinol-binding protein type III

CRBP-III KO – CRBP-III knock-out

CKD – Chronic kidney disease

CLD – Chronic liver disease

Da – Dalton

DGAT1 – Diacylglycerol acyltransferase type 1

eGFR – estimated glomerular filtration rate

ELISA – Enzyme-linked Immunosorbent Assay

ESRD – End-stage renal disease

GLUT4 KO mice – Glucose transporter 4 knock-out mice

HFD – High fat diet

HOMA-IR – Homeostasis Model Assessment of Insulin Resistance

LRAT – Lecithin:retinol acyltransferase

MALDI-TOF-MS – Matrix-assisted laser desorption/ ionisation - time-of-flight - mass spectrometry

MGAT1 – Monoacylglycerol acyltransferase type 1

RA – retinoic acid

RAL – retinaldehyde

RBP4 – Retinol-binding protein 4

RE – retinyl ester

ROH – retinol

RRT – Renal replacement therapy

SD – standard diet

T2DM – Type 2 diabetes mellitus

TTR – Transthyretin

VAD – vitamin A deficient

WAT – white adipose tissue

WHR – Waist-to-hip ratio

WT – wild-type

1 Introduction

1.1 Vitamin A metabolism

Vitamin A belongs to the group of fat-soluble vitamins and exists in several chemical structures such as retinol (ROH), retinylesters (REs), retinaldehyde (RAL) and the biologically most active form retinoic acid (RA) (Gottesman *et al.* 2001). In particular, REs are the storage form of ROH and the biologic active form RA may act in the nucleus as a transcription factor. Since mammals cannot synthesize vitamin A, it is essential, that the diet contains adequate amounts of the vitamin (Goodman 1984; Sporn *et al.* 1994). Dietary vitamin A sources of preformed vitamin A are animal products such as liver, milk and eggs whereas dietary sources of the provitamin A carotenoids such as β-carotene are vegetables and fruits (Goodman 1984).

The retinoids, ROH and its metabolites, are inter alia involved in the process of vision, the immune function, reproduction and embryogenesis. The major end-point of vitamin A deficiency is blindness which is still very common in the developing countries (Desvergne 2007). Vitamin A is also essential for cell proliferation and differentiation (Blaner 2007): For example, after experimental induced myocardial infarction, hepatic ROH is mobilised and delivered to the damaged tissue (Palace *et al.* 1999). The action of vitamin A is accomplished by RA which performs its functions through the binding to nuclear hormone receptors by activating the transcription of certain genes (Chambon 2005; Piantedosi *et al.* 2005; Isken *et al.* 2008).

1.1.1 Absorption, transport and storage

After the digestion of REs or carotenoid containing foods ROH is absorbed from the intestine and is bound to a specific retinol-binding protein (RBP), the cellular retinol binding protein 2 (CRBP2), in the enterocyte. The binding is important, since ROH is hydrophob and can easily be incorporated into membranes and disturb normal cell activities. Therefore RBPs protect the cells against ROH toxicity (Piantedosi *et al.* 2005). CRBP2 facilitates the incorporation of ROH into chylomicrons. Within the chylomicrons ROH is transported to the liver (Ghyselinck *et al.* 1999) where these are taken up by the liver cells. Within the hepatocytes ROH is bound to cellular retinol binding protein type I (CRBP-I) which transports ROH to the hepatic stellate

cells. There it is esterified with long chain fatty acids to its main RE, retinyl palmitate, and stored. This process is mainly catalysed by the enzyme lecithin:retinol acyltransferase (LRAT) in the liver and the eye and by the enzymatic activity called acyl-CoA: retinol acyltransferase (ARAT) (Ghyselinck *et al.* 1999; O'Byrne *et al.* 2005). To that date the molecular identification and characterization of the enzyme carrying out the ARAT reaction in the liver and extra-hepatic tissues remains unclear (O'Byrne *et al.* 2005). The liver is the central organ in ROH metabolism, storing and releasing ROH in dietary excess and deficiency, respectively (Ghyselinck *et al.* 1999). The release of ROH from the liver is triggered by the binding of retinol-binding protein 4 (RBP4) to ROH and the additional binding of this complex to transthyretin (TTR). TTR is therefore an indirect ROH transport protein and protects the RPB4-ROH complex of being filtered by the renal glomerulus. Within the complex ROH is transported to its target tissues e.g. the eye where RBP4 binds to its receptor (Stimulated by Retinoic Acid 6, STRA6) and ROH is transferred into the target cell (Kawaguchi *et al.* 2007). The remaining RBP4, which is then called apo-RBP4, is rapidly catabolised in the kidneys (Gerlach and Zile 1991). Through the reabsorption of ROH and RBP4 in the proximal tubular system, the kidneys are known to maintain whole body ROH homeostasis (Raila *et al.* 2007). REs – in contrast to ROH, which is bound to RBP4 – are transported via lipoproteins in the serum which account for 5% of total plasma ROH (Mills *et al.* 2008).

1.1.2 Cellular actions of vitamin A and its metabolites

Within the cytoplasm of target cells ROH is bound to its cellular retinol-binding proteins (CRBPs), namely CRBP-I, CRBP-II and CRBP-III[1] (Noy 2000; Zizola *et al.* 2008). CRBPs bind ROH and mediate esterification and facilitate the oxidation to the intermediate RAL and to the major biologically active end-product RA (Zizola *et al.* 2008). RA regulates gene expression through its binding to the nuclear hormone receptors retinoic acid receptor (RAR) and the retinoid X receptors (RXR) (Blaner 2007). The enzymes alcohol dehydrogenase (ADH) and retinaldehyde dehydrogenase (RALDH) catalyse the oxidation of ROH to RA (Figure 1).

Next to the liver, the white adipose tissue (WAT) may store about 20% of body retinoids as REs and the per cell retinoid content is twice as much as in hepatic

[1] The CRBPs in this work all refer to the murine CRBP family. For CRBP-III it has to be noted that he murine CRBP-III is not homologous to the human CRBP-III.

stellate cells (Tsutsumi *et al.* 1992; Yang *et al.* 2005; Yoshida *et al.* 2006). However, adipose tissue is also a target for retinoids as these may modulate adipose structure and its metabolic function through their binding to the nuclear hormone receptors RAR and RXR. In fact, by binding to the ligand binding domain of RAR or RXR RA can inhibit or enhance adipogenesis, respectively (Ziouzenkova and Plutzky 2008). However, to that date, metabolism of ROH concerning the storage and action in adipose tissue remains unclear. The intracellular metabolism of vitamin A and its metabolites is exemplified in Figure 1.

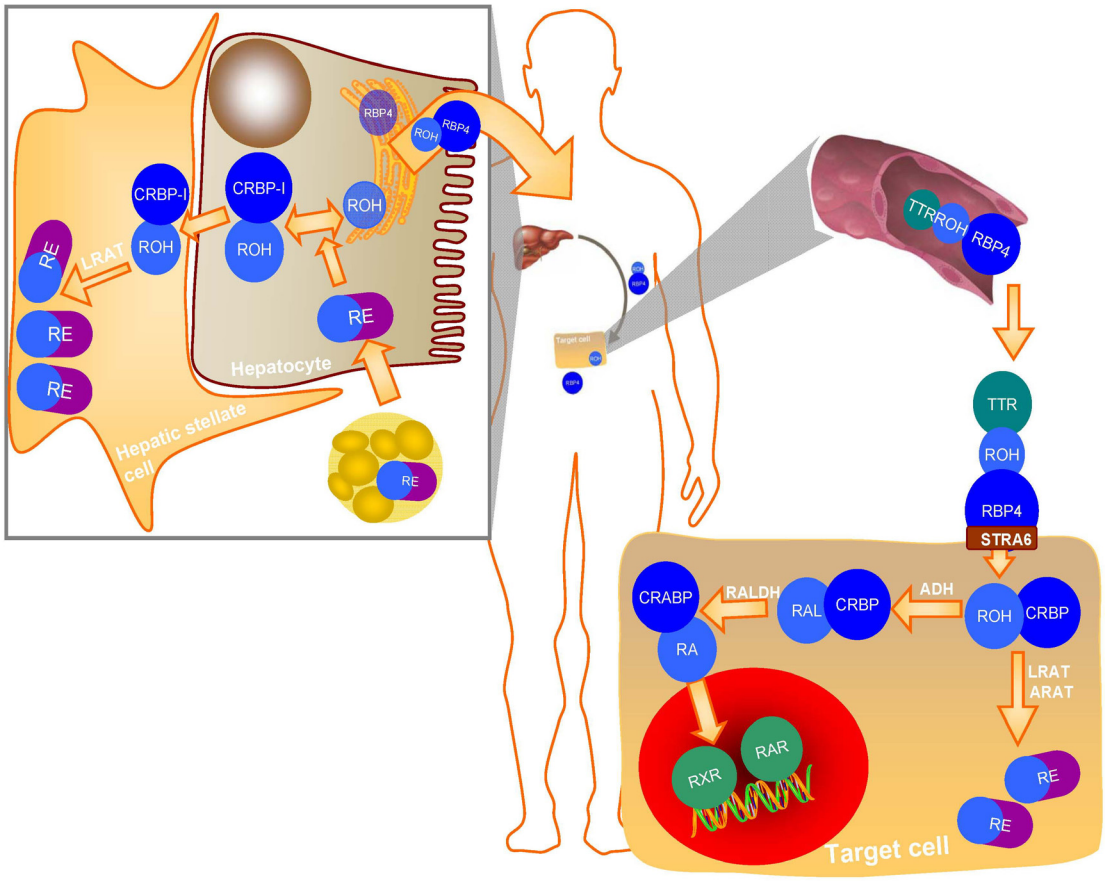

Figure 1 Intracellular metabolism of retinol (ROH).
ROH is transported in a complex with RBP4 and TTR to the target cells where it binds to the receptor Stimulated by Retinoic Acid 6 (STRA6). Within the cell ROH is bound to cellular retinol binding proteins (CRBP) and may be oxidised to retinaldehyde (RAL) and retinoic acid (RA). The reactions are catalysed by alcohol dehydrogenase (ADH) and RAL dehydrogenase (RALDH). RA may activate the nuclear receptors RXR and RAR and induce the transcription of certain genes.

1.2 Extracellular retinol-binding proteins

In the circulation ROH is transported via RBP4 and TTR. The next two chapters will provide insights in the physiology of these proteins. Both proteins share the attribute to be synthesised in the liver and are therefore useful proteins to assess nutritional status (Yoshida *et al.* 2006).

1.2.1 Retinol-binding protein 4 (RBP4)

For retinoid-dependent processes (cell differentiation and growth) it is important that cells and tissues are supplied sufficiently with ROH. In the blood stream RBP4 ensures the supply of ROH from the liver throughout the body (Isken *et al.* 2008). RBP4 has a molecular weight of approximately 21.000 Dalton and belongs to the protein family of the lipocalins[2] which is known to bind hydrophobic ligands (Lewis *et al.* 2007). Since the protein was isolated in 1968 (Kanai *et al.* 1968) a huge amount of experiments on its structure, metabolism and physiological roles have been carried out.

The main site of RBP4 expression is the liver. However, the adipose tissue has the second highest expression level and the kidney about 10% of the expression in the liver (Yang *et al.* 2005; Mills *et al.* 2008). RBP4 expression in the liver is regulated by the availability of vitamin A and nutritional status (Lewis *et al.* 2007). In healthy states ROH binds to RBP4 (holo-RBP4) at the entry of the Golgi apparatus, from where it is secreted in a complex with a second protein: TTR (Noy 2000; Selvaraj *et al.* 2008). The whole process is triggered by the presence of ROH (Ronne *et al.* 1983). Due to the binding to TTR the RBP4-ROH complex is prevented from renal excretion (Goodman 1980). During liver dysfunction and inflammation the synthesis of RBP4 is reduced and therefore RBP4 serum levels decrease (Smith and Goodman 1971; Schweigert 2001). During vitamin A deficiency RBP4 accumulates in the endoplasmatic reticulum of the hepatocyte, since the secretion but not the synthesis is dependent on ROH (Ronne *et al.* 1983). In the target tissues RBP4 binds a membrane-bound receptor which has been identified to be STRA6 and which facilitates the cellular ROH uptake (Blaner 2007; Kawaguchi *et al.* 2007). After the release of ROH into the target cell, the RBP4-TTR complex dissociates and the remaining RBP4 (apo-RBP4) is rapidly transported to the kidneys where it is

[2] Members of the lipocalin-family are retinoid-binding proteins, epididymal retinoic acid-binding protein and beta-lactoglobulin. The members show a low sequence identity but have a highly conserved overall fold. Noy, N. (2000). "Retinoid-binding proteins: mediators of retinoid action." Biochem J **348 Pt 3**: 481-95.

filtered through the glomeruli and reabsorbed in the proximal tubule cells. During kidney dysfunction RBP4 levels increase due to a loss of functional renal tissue (Stewart and Fleming 1982). Depending on the interaction with the ligand ROH, RBP4 exists therefore in its holo- (bound to ROH) and apo-form (not bound to ROH) (Noy *et al.* 1992; Zanotti and Berni 2004).

Moreover, little is known about structural modifications in the RBP4 molecule. It has been reported during the 90ies, that in the serum of patients with chronic kidney disease (CKD), the RBP4 molecule may be altered and occurs in two isoforms in human serum: First, truncated at the C-terminal end by one leucine (Leu-183) and, second at the C-terminal end by two leucine molecules (Leu-182), named RBP4-L and RBP4-LL, respectively Figure 2. It has been speculated, that these truncated RBP4 forms are rapidly cleared by the kidneys in healthy subjects, and are thus only present under certain pathophysiological conditions (Jaconi *et al.* 1995; Jaconi *et al.* 1996).

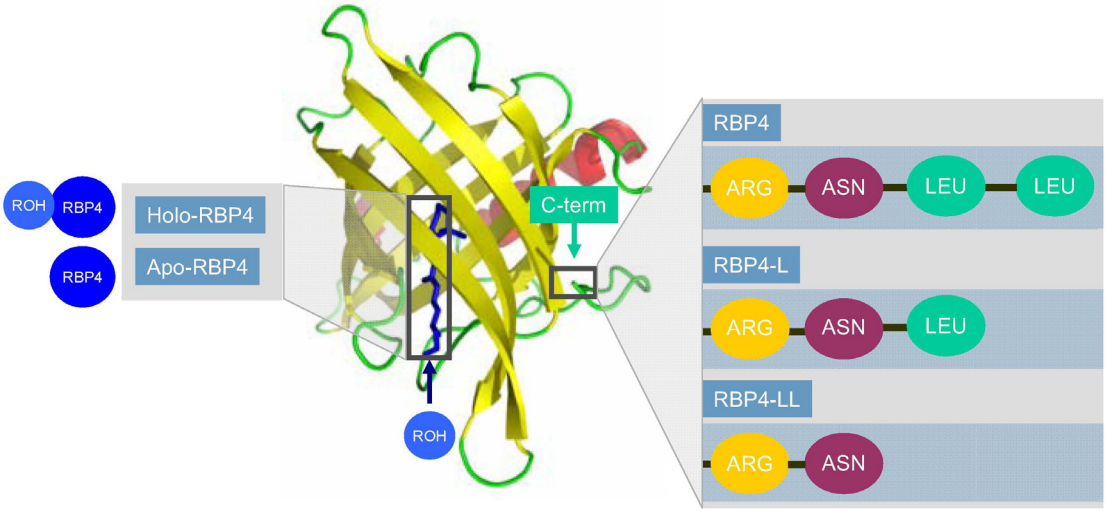

Figure 2 Molecular structure of retinol-binding protein 4 (RBP4).
RBP4 bind retinol (ROH) and is then called holo-RBP4. After the release of ROH into target cells the remaining RBP4 is called apo-RBP4. The C-terminal end of the protein is characterised by the amino acids asparagin (ASN) and two leucine (LEU) molecules. RBP4-L and RBP4-LL are truncated by one and two LEU molecules, respectively.

Taken together, the main function of RBP4 is to mobilize ROH from hepatocytes and to transport it to target cells. During liver and kidney dysfunction RBP4 serum levels are affected (Whaley-Connell *et al.* 2008).

1.2.2 Transthyretin (TTR)

TTR[3] is a homotetrameric protein with approximately 14.000 Dalton per monomer and binds the thyroid hormone thyroxin (T4). Moreover, TTR is involved in the transport of ROH in the blood through the binding to RBP4 (Zanotti and Berni 2004). Since the TTR tetramer provides a molecular weight of approximately 56.000 Da, RBP4 is – by the binding to TTR – protected from glomerular filtration (Sousa *et al.* 2000) and ensures thus the transport of ROH to the target tissues (Noy 2000). About 50% of TTR are bound to RBP4 in healthy subjects, whereas in patients undergoing haemodialysis (HD) the amount of TTR (in particular the amount which is bound to RBP4) increases (Cano *et al.* 1988). TTR is mainly synthesised in the liver and its serum levels are affected during liver dysfunction, inflammation and malnutrition (Smith and Goodman 1971; Filteau *et al.* 2000; Schweigert 2001).

It has also been suggested that structural modifications of TTR, which diagnostically can be identified as TTR isoforms, might affect the binding affinity of TTR to RBP4 and thus the transport of ROH to target tissues. Each TTR monomer has a single cysteine residue in position 10 that can exist in the native form with a free sulfide group or as a mixed disulfide with the amino acid cysteine (cys-TTR) as well as the peptides cysteinylglycine (cysglyc-TTR) and glutathione (glut-TTR) (Gericke *et al.* 2007). Although it is not known whether specific TTR isoforms preferentially bind to RBP4, the binding of TTR to RBP4 involves the C-terminal end of the RBP4 molecule (Noy 2000). Moreover, the interaction between RBP4 and TTR is also influenced by the amount of holo-RBP4 (ROH-bound) and apo-RBP4 (ROH-free) (Peterson 1971).

[3] The protein has been called prealbumin since the Nomenclature Committee of IUB and the IUPAC-IUB Joint Commission on Biochemical Nomenclature suggested transthyretin in 1981.

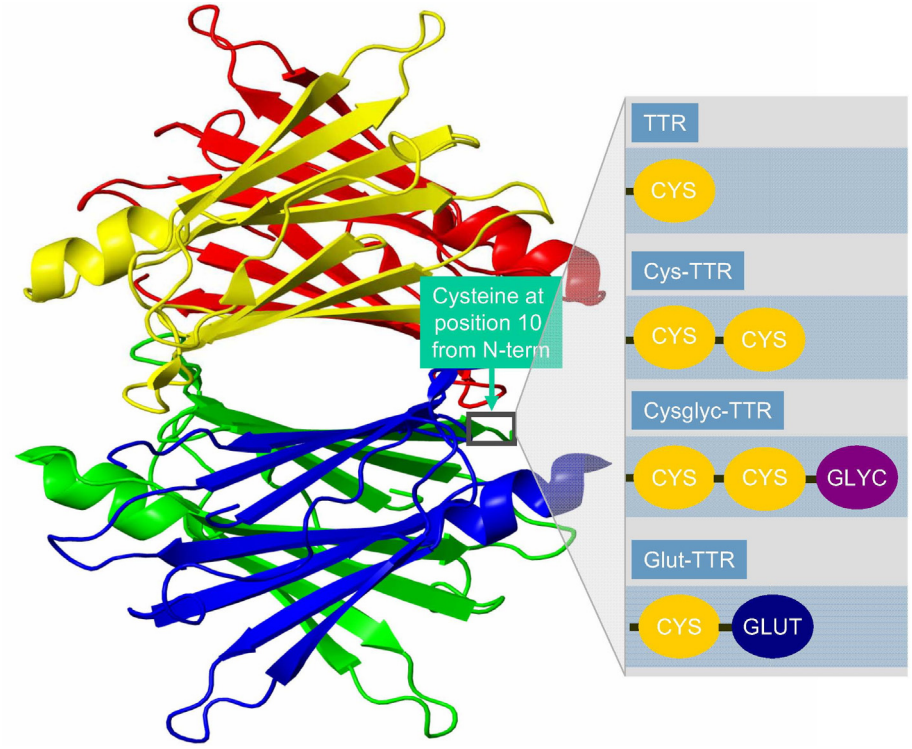

Figure 3 Molecular structure of the tetramer transthyretin (TTR).
The TTR tetramer is made up of four subunits based on one TTR molecule (yellow, red, blue, green). At position 10 (N-terminal end) of every subunit of the TTR amino acid chain a cysteine residue is located. The sulfide group of the cysteine is able to form a mixed disulfide with a second cysteine (Cys-TTR), with the peptide cysteinylglycine (Cysglyc-TTR) or a glutathione molecule (glut-TTR).

To that date, it is known that in amyloidosis TTR may undergo structural rearrangement, aggregation, and deposition and may cause organ dysfunction. It was speculated that the underlying structural instability of TTR could be due to post-translational modifications of TTR, i.e. TTR isoforms (Kingsbury *et al.* 2008).

1.3 Intracellular retinol-binding proteins

Within the cells ROH is bound to CRBPs. These proteins belong to the family of fatty acid binding proteins and are known to bind their ligands noncovalently (Vogel *et al.* 2001). Due to the binding of ROH to CRBPs their transport to its metabolizing enzymes is facilitated and thus the esterification of ROH to RE in hepatic stellate cells. In addition, through the binding to RBP4 cells are protected against ROH toxicity (Piantedosi *et al.* 2005; Zizola *et al.* 2008).

1.3.1 Cellular retinol-binding protein type I (CRBP-I)

CRBP-I has been discovered in 1973 and since that date its localization, expression and structure have been studied (Ghyselinck *et al.* 1999). CRBP-I is highly expressed in the liver, kidney, heart, muscle and brain (Noy 2000; Piantedosi *et al.* 2005) and also in WAT (Zizola *et al.* 2008). The main function of CRBP-I is to transport ROH to its metabolizing enzymes for esterification to RE or oxidation to RA (Ghyselinck *et al.* 1999). Therefore, CRBP-I knock-out (CRBP-I KO) mice show reduced hepatic retinyl palmitate (main ester of ROH) stores but do not show signs of ROH deficiency if maintained on a vitamin A containing diet (Ghyselinck *et al.* 1999). In contrast, if maintained on a retinoid-insufficient diet CRBP-I KO mice show signs of hypovitaminosis A earlier compared to wild-type (WT) mice (Piantedosi *et al.* 2005). CRBP-I expression increases when intracellular levels of ROH are low thereby increasing the uptake of ROH into the cell (Noy 2000). The main ROH esterifying enzyme in the liver is lecithin:retinol acyltransferase (LRAT). Although CRBP-I is also synthesised in extra-hepatic tissues such as the adipose tissue, the function of CRBP-I in these tissues remains unclear. In fact, the process of esterification of ROH in extra-hepatic tissues is known to be mediated by the enzymatic activity ARAT but the enzyme behind the process is unclear (Ghyselinck *et al.* 1999; Piantedosi *et al.* 2005). In addition, by its binding to ROH it protects the cells of the membranolytic effects of ROH (Noy 2000). The concentration of ROH is always lower than that of CRBP-I, as free ROH is toxic for cells (Ghyselinck *et al.* 1999).

1.3.2 Cellular retinol-binding protein type II (CRBP-II)

CRBP-II is mainly expressed in the small intestine and is involved in retinoid incorporation into chylomicrons (Ghyselinck *et al.* 1999; Noy 2000; Piantedosi *et al.* 2005). The protein is not further considered in this work.

1.3.3 Cellular retinol-binding protein type III (CRBP-III)

CRBP-III was discovered in 2001 and belongs – like CRBP-I and CRBP-II – to the family of fatty acid binding proteins (Vogel *et al.* 2001). CRBP-III is highly expressed in tissues with little or no CRBP-I expression. In adipose and mammary tissue both proteins are expressed, whereas no CRBP-III is found in the liver. CRBP-III binds ROH, but not RAL, in the cytosol (Piantedosi *et al.* 2005). This is in contrast to CRBP-I and CRBP-II which both also bind RAL. To that date it is believed

that the main function of CRBP-III is to facilitate ROH esterification in the mammary gland during lactation (Piantedosi *et al.* 2005). Mice lacking CRBP-III (CRBP-III KO) are viable and healthy but show a decreased amount of retinoids in the milk (Piantedosi *et al.* 2005).

Apart from this, its physiologic role is still under consideration. In fact, it has been shown that CRBP-III KO mice maintained on a high-fat diet (HFD) remain more glucose tolerant and accumulate less triglycerides in the liver compared to wild-type (WT) mice. This is partly due to a lower release of free fatty acids from the adipose tissue in the circulation (Zizola *et al.* 2008). Moreover, CRBP-III is expressed in both adipocyte cell types, adipocytes and stromal vascular cells. Since CRBP-I is entirely expressed in stromal vascular cells one might assume that the proteins have different physiological roles within adipose tissue (Vogel *et al.* 2001).

In summary, both, extra- and intracellular retinol-binding proteins, solubilize and stabilize their hydrophobic ligand ROH in the aqueous space and are important for the regulation of ROH transport, metabolism and action (Noy 2000).

1.4 Obesity, type 2 diabetes and related organ dysfunctions

The prevalence of obesity and related diseases such as adiposity and type 2 diabetes mellitus (T2DM) is strongly increasing, especially in developed countries (Ritz *et al.* 1999; Guilherme *et al.* 2008). As a result dysfunctions of the kidney (diabetic nephropathy) and the liver (fatty liver) are very common. The reasons for the development of obesity and type 2 diabetes and the relation to organ dysfunctions will be regarded further in the next chapters.

1.4.1 Obesity and the development of type 2 diabetes

Obesity is defined as body mass index (BMI) between 25 and 30 kg/m^2 and adiposity as a BMI greater than 30 kg/m^2 (Guilherme *et al.* 2008). Both, obesity and T2DM, are accompanied by glucose intolerance, and often by hypertension and dyslipidemia. The combination of these disorders is outlined as "metabolic syndrome" being a major risk factor for T2DM and cardiovascular diseases (Eckel *et al.* 2005). T2DM is characterised by peripheral insulin resistance, impaired regulation of hepatic glucose production and declining beta-cell function (Mahler and Adler 1999; Lewis *et al.* 2002). Patients suffering from T2DM do not have an absolute insulin deficiency, but rather a peripheral insulin resistance which is, at

least at the beginning, accompanied by an increased production of insulin by endocrine pancreatic cells (Mahler and Adler 1999). Liver, skeletal muscle and adipose tissue are major targets for the metabolic actions of insulin (Tamori *et al.* 2006).

For a long time adipose tissue has simply been regarded as storage site for triglycerides in the body. During the last decade, the functions of the WAT did enlarge due to the discovery of adipokines – proteins, which are secreted by the adipose tissue. They are involved in regulating body weight, energy homeostasis and insulin sensitivity (Zizola *et al.* 2008).[4] In obesity, the average adipocyte cell size increased due to the accumulation of triglycerides in the lipid droplet. Moreover, macrophages infiltrate the adipose tissue and both, adipocytes and macrophages secrete adipokines such as adiponectin, visfatin and leptin into the circulation. These proteins may mediate insulin resistance (Kloting *et al.* 2007; Wolf 2007). The consequence of insulin resistance is characterised by a decreased uptake of glucose in the muscle and adipose tissue and the failure of insulin to suppress hepatic glucose production (Farrell and Larter 2006). Normal insulin sensitivity and glucose homeostasis require a functional adipose tissue which is in proper size to whole body weight (Guilherme *et al.* 2008). Due to an increase in adipokine secretion in obesity, the incidence of insulin resistance increased along with an increased lipolysis from the WAT. Consequently, serum levels of free fatty acids and glycerol rise during obesity thereby enhancing insulin resistance and the prevalence for T2DM (Lewis *et al.* 2002; Villarroya *et al.* 2004).

1.4.2 Obesity, type 2 diabetes and the relation to kidney dysfunction

Obesity, i.e. an increased BMI due to an increase in fat mass, has been shown to be an independent predictor of end-stage renal failure (ESRF) also after adjustment for confounding factors such as blood pressure. BMI is thus strongly related to kidney function (Axelsson 2008; Ritz 2008). In countries with a western lifestyle the incidence of patients suffering from ESRF and T2DM has increased steadily in the last decades and diabetes mellitus has become the major cause of ESRF. Simultaneously, the amount of diabetic patients with ESRF receiving renal replacement therapy (RRT) such as haemodialysis (HD) or renal transplantation (RTx) did increase (Ritz *et al.* 1999): Data of *The US Renal Data System* reveal that

[4] This is in contrast to brown adipose tissue which uses excess energy for the production of heat. Desvergne, B. (2007). "Retinaldehyde: more than meets the eye." Nat Med **13**(6): 671-3.

approximately 40% of receiving RRT patients suffer also from diabetes mellitus and that 69% of these patients are treated by HD and 18% by RTx (Ritz *et al.* 1999).

The main function of the kidney is to regulate the body's acid-base balance and the blood pressure as well as to catabolize serum proteins such as albumin and RBP4. The kidney reabsorbs glucose and proteins in its tubular system and also excretes creatinine in the urine (Mutschler *et al.* 2007). Renal dysfunction is characterised by an increase in serum levels of small proteins (Mogielnicki *et al.* 1971; Vahlquist *et al.* 1973). Interestingly, renal dysfunction itself increases insulin resistance (Chang *et al.* 2008) due to the decreased renal clearance of adipokines thereby increasing serum levels (Axelsson 2008). Apart from having effects on the glucose and insulin homeostasis, adipokines also influence endothelial function and may increase the risk of cardiovascular disease (Axelsson 2008). Morbidity and mortality in patients suffering from chronic kidney disease (CKD) are mainly due to the high prevalence of cardiovascular disease (Ritz *et al.* 1999; Axelsson 2008).

The interesting fact between T2DM and kidney dysfunction is, that on the one hand, insulin resistance and diabetes mellitus which are present in the majority of CKD patients may initiate diabetic nephropathy. On the other hand, kidney dysfunction itself increases insulin resistance (Axelsson 2008).

1.4.3 Obesity, type 2 diabetes and the relation to liver dysfunction

The functions of the liver are various. To name a few it synthesizes hormones, serum proteins such as albumin and lipoproteins. Furthermore it stores triglycerides and glycogen. On the other hand, the liver is responsible for the catabolism of amino acids, triglycerides and may transform free fatty acids in ketons (Mutschler *et al.* 2007). In times of excess caloric intake an increased triglycerides uptake in the adipocytes leads in turn to an increased release of free fatty acids into the circulation from adipose tissue (Guilherme *et al.* 2008). Due to that, the increased lipogenesis in the liver exceeds the capacity of the hepatocytes to export very low-density lipoproteins and leads to the accumulation of triglycerides in the hepatocyte (Lewis *et al.* 2002; Wu *et al.* 2007). As a consequence, lipid droplets are stored in the cytosol of liver cells. This may – on the one hand – contribute to the development of insulin resistance and – on the other hand – lead to the development of fatty liver (Bugianesi *et al.* 2005). Therefore, one risk factor for

chronic liver diseases (CLD) is obesity. In fact, both, T2DM and obesity increase the risk for the clinicopathologic condition of non-alcoholic fatty liver disease (NAFLD). Within the last ten years *in vitro* and clinical studies did reveal that NAFLD is associated with a wide spectrum and more severe forms of liver dysfunction such as simple steatosis, steotohepatitis and fibrosis which may progress to cirrhosis and also hepatocellular carcinoma (McCullough 2002; Farrell and Larter 2006).

1.5 Retinol-binding proteins (RBPs) in obesity and related diseases

1.5.1 Introduction

The main function of RBP4 has been known to be the transport of ROH in the serum (Goodman 1984). In an article published in *NATURE* in 2005 (Yang *et al.* 2005), RBP4 has been postulated to contribute to insulin resistance and has thus become a new field of research. Based on that study an emerging amount of experiments in humans and mice has been carried out, but still the mechanisms behind the relation of RBP4 and T2DM remain unclear. This is due to a complex play of several factors that are involved in RBP4 and ROH metabolism (Figure 4).

1.5.2 Extracellular RBPs in obesity and type 2 diabetes

Yang and Graham (2005) showed in their study a correlation between increased RBP4 serum levels and the incidence of T2DM and insulin resistance. Their results are based on findings in mice in which the glucose transporter 4 (GLUT4) was selectively knocked out (GLUT4 KO mice) in adipose tissue. GLUT4 is involved in the transport of glucose from the circulation into muscle and adipose tissue. During the development of insulin resistance the amount of GLUT4 decreases. In the GLUT4 KO mice it has been shown that the expression of RBP4 is induced in adipocytes thereby increasing serum RBP4 levels (Yang *et al.* 2005). It has thus been speculated that adipocyte derived RBP4 – not hepatic RBP4 – may induce insulin resistance (Yang *et al.* 2005; Zizola *et al.* 2008). In that context it was shown that chronic RBP4 elevation did increase hepatic glucose production through a stimulation of the hepatic enzymes (e.g. phosphoenolpyruvate carboxykinase) and did downregulate insulin signalling in the muscle (Goodman *et al.* 2008).

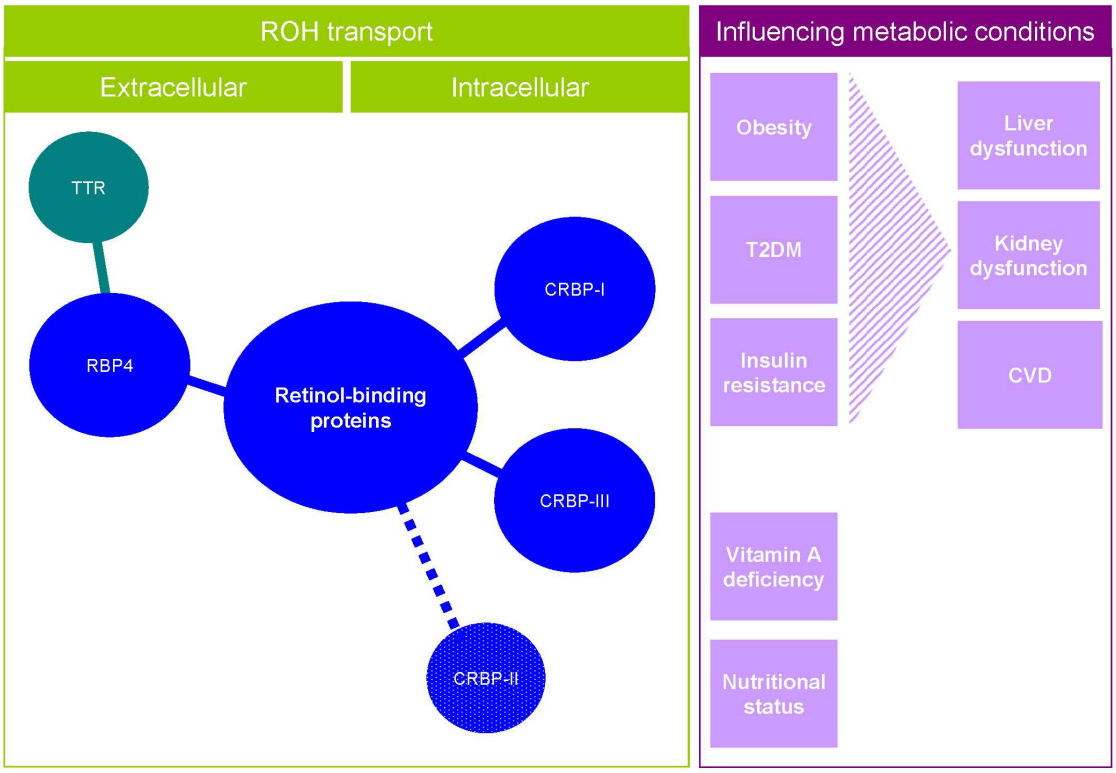

Figure 4 Overview of Retinol-binding proteins and of metabolic conditions known to interfere with retinol (ROH) homeostasis and its binding proteins.
ROH supply to cells is mediated through retinol-binding protein 4 (RBP4) and transthyretin (TTR) in serum, i.e. extracellular. Within the cell ROH is bound to cellular retinol-binding protein type I, 2 or 3 (CRBP-I, CRBP2, CRBP-III). CRBP-I facilitates hepatic ROH conversion to retinyl esters (RE) whereas CRBP2 regulates intestinal ROH absorption in the enterocyte. CRBP-III is known to facilitate RE formation in the mammary gland and is highly expressed in adipose tissue. Nutritional status, vitamin A deficiency, liver and kidney dysfunction are classical parameters influencing ROH and RBP4 levels in serum. Newly discovered conditions interfering with ROH and RBP4 are obesity, type 2 diabetes (T2DM) and cardiovascular disease (CVD).

In addition, it was suggested that RBP4 would inhibit the phosphorylation of the insulin receptor (Ziegelmeier *et al.* 2007) and that RBP4 might influence RXR, the nuclear receptor for RA, and therefore glucose homeostasis (Tamori *et al.* 2006). To that date the results are still conflicting as some studies confirmed this relation (Graham *et al.* 2006; Aeberli *et al.* 2007; Qi *et al.* 2007) and others did not (Janke *et al.* 2006; Broch *et al.* 2007; Lewis *et al.* 2007; von Eynatten *et al.* 2007; Ziegelmeier *et al.* 2007; Lewis *et al.* 2008).

With regard to RBP4, it is unclear whether apo- or holo-RBP4 contributes to insulin resistance. As holo-RBP4 is known to possess a higher binding affinity to TTR, it is supposed that an increase in holo-RBP4 might contribute to a decreased clearance of RBP4 from serum and that holo-RBP4 may thus account for elevated RBP4 levels in systemic insulin resistance (Mody *et al.* 2008). However, apo-RBP4 levels were

increased in overweight subjects (Mills *et al.* 2008). Apart from that, it has been shown that TTR levels are increased in subjects with impaired glucose tolerance and with T2DM (Kloting *et al.* 2007).

1.5.3 Extracellular RBPs in kidney dysfunction

Since research on RBP4 has been carried out, it has become obvious that RBP4 serum levels increase during kidney dysfunction and that kidney function does strongly determine RBP4 serum levels. This is due to the decreased ability of the glomerulus to filter low-molecular weight proteins (Jaconi *et al.* 1996). Along with that, a decreased catabolism due to tubular dysfunction enhances renal excretion of small serum proteins, such as RBP4 (Smith and Goodman 1971). For a long time, CKD and ESRF have been the only known clinical conditions in which serum levels of RBP4 are chronically elevated (Kelleher *et al.* 1983; Schöne 1983) and thus RBP4 has been discussed as a marker for renal function (Donadio *et al.* 2001; Cabre *et al.* 2007; Ziegelmeier *et al.* 2007). In addition to that, apo-RBP4 levels are increased in the serum of patients with CKD indicating that the catabolism and reabsorption of RBP4 in the kidneys is disturbed (Gerlach and Zile 1991; Jaconi *et al.* 1996). The interesting fact is, that renal dysfunction is very common in T2DM patients and leads to an increase of RBP4 levels in the circulation (Ziegelmeier *et al.* 2007). Even in a study carried out by this institute, T2DM patients with minor kidney dysfunctions – assessed by the excretion of albumin in the urine – showed elevated RBP4 levels compared to controls (Raila *et al.* 2007).

Beside the elevation of RBP4 levels during kidney dysfunction the relative amounts of RBP4-L and RBP4-LL increase (Jaconi *et al.* 1996). It has been speculated that these truncated RBP4 forms are rapidly cleared by the kidneys in healthy subjects and are thus only present under certain pathophysiological conditions (Jaconi *et al.* 1995; Jaconi *et al.* 1996). To that date, the influence and metabolic conditions of kidney dysfunction on the occurrence of RBP4 isoforms remain unknown.

With regard to TTR, the protein is – due to its intermediate size of 56 kDa – not able to pass the glomerular barrier and its metabolism is therefore only to a small extent dependent on the function of the kidney (Vahlquist *et al.* 1973). Therefore, TTR has been shown to be a reliable nutritional marker in HD patients (Ingenbleek, et al., 1975). However, in dialysis patients positive inflammation markers (such as CRP) are increased and lead in turn to a decrease in TTR levels. Moreover, a

reduction of TTR serum levels in HD patients is mainly linked to the situation of malnutrition and inflammation during ESRF. To that date, the effect of kidney dysfunction on TTR isoforms has not been studied.

1.5.4 Extracellular RBPs in liver dysfunction

During liver dysfunction ROH, RBP4 and TTR levels are affected, i.e. decreased and are therefore important predictors of the functional status of the liver. It is known that in liver cirrhosis, RBP4 and TTR levels are decreased due to a loss of functional tissue. Interestingly, chronic liver disease (CLD) is associated with alterations in glucose metabolism leading to impaired glucose tolerance and T2DM (Bahr *et al.* 2008). Moreover, since RBP4 and TTR are negative acute phase proteins their hepatic synthesis is also decreased during inflammation (Gruys *et al.* 2005) and during malnutrition (Ingenbleek *et al.* 1975; Cano *et al.* 1988). Alterations in RBP4 and TTR levels during CLD have been studied, whereas RBP4 and TTR isoforms have not been investigated so far.

1.5.5 Intracellular RBPs and obesity

To that date knowledge on CRBPs in obesity and their function in adipose tissue is still limited. In fact, the functions of CRBP-I in the liver have been studied extensively whereas extra-hepatic functions of CRBP-I – although it is known that the protein is expressed in adipose tissue and pancreas – need to be elucidated.

With regard to CRBP-III, it has been shown that CRBP-III knock-out (CRBP-III KO) mice maintained on a HFD have a reduced food intake with a decreased adiposity compared to WT mice. In addition, the knock-out mice showed decreased hepatic steatosis compared to wild-type mice (Zizola *et al.* 2008). This is mainly due to the decreased lipolysis in WAT of CRBP-III KO mice resulting in decreased serum levels of free fatty acids. In addition, it has been shown that CRBP-III expression was increased in WAT after treatment with rosiglitazone, a PPARγ agonist.

2 Aim

Retinoids, ROH and its metabolites, have been reported to be involved in adiposity and energy metabolism through their effect on adipocyte differentiation and on energy expenditure genes. Therefore they have been postulated to be new players in the context of obesity and T2DM. Their physiological action is dependent on adequate ROH supply to target tissues and on a regular intracellular metabolism. In detail, ROH metabolism is dependent on the availability of RBPs on extracellular level – the site of ROH supply – and on intracellular level – the site of ROH demand. In fact, the reported elevation of RBP4 levels under insulin-resistant conditions might be the result of altered RBP4 secretion from adipose tissue, of altered RBP4 clearance from the circulation or an increased ROH demand on intracellular level. The aim of this thesis is therefore to investigate the relation of RBPs and its ligand ROH on extra and intracellular level. Figure 5 illustrates the structure of the work:

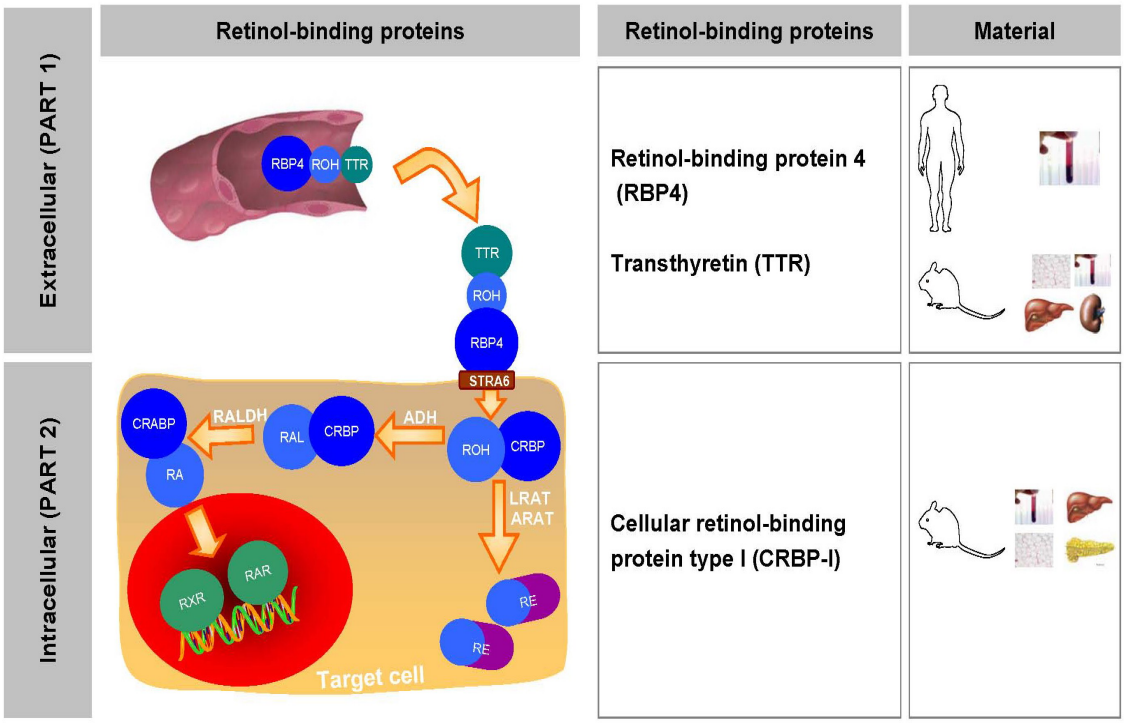

Figure 5 Overview of the structure of the thesis
Part 1 investigates retinol-binding protein 4 (RBP4) and transthyretin (TTR) which transport retinol (ROH) to target cells in human and murine samples. Part 2, investigates cellular retinol-binding protein type I (CRBP-I) which mediates the intracellular ROH metabolism to retinylesters (RE) by the enzymes LRAT/ARAT or to retinoic acid (RA) by the enzymes ADH/RALDH. RA itself is able to bind to the nuclear hormone receptors (RAR or RXR) and to induce the transcription of certain genes.

RBP4, the most common member of ROH binding proteins, had been deemed only a transport medium for vitamin A in the blood stream until scientists discovered a relation between RBP4 and T2DM in 2005. However, this relation is affected by the influence of several factors that may influence RBP4 levels such has the availability of ROH, nutritional status, liver and kidney function and modifications of the molecular structure of RBP4. These influencing factors have not been investigated within the context of obesity and T2DM and there is still considerable scientific debate about these findings. The first part of this work therefore focuses on the extracellular RBPs, RBP4 and TTR (Figure 5). In detail, this part investigates the following items:

Studies in mice

- The adipose tissue, kidneys and the liver are central organs of ROH metabolism. Therefore tissue specific alterations as well as the location of RBP4 and TTR in white adipose tissue, kidney and liver in obese mice were investigated.
- Although the regulation of RBP4 expression in the liver has been studied extensively, the regulation in the adipose tissue remains unclear. Therefore experiments on the regulation and secretion of RBP4 and TTR after stimulation of mouse adipocytes have been performed.
- RBP4 serum levels in relation to kidney function as a major influencing factor have been investigated in diabetic *db/db* mice.

Clinical studies

- In patients with obesity and T2DM serum RBP4 and TTR levels have been investigated. Moreover specific alterations in the molecular structure of human RBP4 and TTR were evaluated.
- Since RBP4 metabolism is affected by certain pathophysiological states such as kidney and liver dysfunction, the impact of these dysfunctions on RBP4 and TTR levels and isoforms were investigated in patients with kidney dysfunction undergoing haemodialysis, in patients after renal transplantation and in patients suffering from chronic liver disease.

In addition, little is known about the intracellular transport and metabolism of ROH – the site of ROH demand – in extra-hepatic tissues. The intracellular metabolism of ROH is of interest since ROH is indirectly involved in specific processes such as adipogenesis and insulin secretion and sensitivity by activating its nuclear

receptors. Since the knowledge about the functions of CRBP-I remains limited on the liver, the aim of the second part of this thesis is to investigate the functions of CRBP-I in extra-hepatic tissues. Therefore mice with a genetic deletion of CRBP-I were generated to examine:

- ROH and RE levels in the liver and extra-hepatic tissues such as adipose tissue, pancreas and lung in CRBP-I KO and WT mice,
- ROH and RE levels after maintaining CRBP-I KO and WT mice on a vitamin A deficient diet
- postprandial ROH uptake into hepatocytes, adipocytes and pancreatic cells,
- and to evaluate body composition and parameters of the glucose and lipid homeostasis.

3 Materials and Methods

3.1 Materials

3.1.1 Animals and diets

WT mice on standard diet or high fat diet and ob/ob mice

WT (C57BL/6J, Jackson Laboratory, Maine, USA) and *ob/ob* (C57BL/6J *Lep*^{ob,} Jackson Laboratory, Maine, USA) mice were obtained from the Department of Preventive Medicine, Columbia University, New York, USA. Mice were placed on a SD (PicoLab® Rodent Diet 20, St. Louis, USA) or HFD (Research Diets Inc, New Brunswick, USA) providing 10% and 60% of calories from fat, respectively. After 40 weeks, mice were sacrificed, the liver perfused with phosphate buffered saline (PBS) and tissues excised, flash frozen in liquid nitrogen and stored at -70°C until analysis. Experiments involving mice were performed with the approval of the Institutional Animal Care and Use Committee at Columbia University.

***Serum of db/db* mice**

Serum of diabetic *db/db* mice was obtained from the Department of Pharmacology, German Institute of Human Nutrition, Potsdam-Rehbrücke. Male *db/db* mice (BKS.Cg-m+/+Leprdb/J) and lean C57BL/KsJ litter mates (BKS.Cg-m+/+/J) (Charles River, Sulzfeld, Germany) were housed in groups of 5 per cage at a temperature of 21 ± 1 °C with a 12:12 hours light-dark cycle. Animals had free access to food and water. All experiments were approved by the Ethics Committee of the State Ministry of Agriculture, Nutrition and Forestry (State of Brandenburg, Germany). All diets were purchased from Altromin (Lage, Germany). The SD (Art. No. C 1000) contained (w/w) 5.1% fat and the HFD (Art. No. C1057) contained (w/w) 14.6% fat. The dietary intervention was started at the age of 5 weeks and continued for 22 weeks or until the development of severe hyperglycaemia (blood glucose > 25 mmol/l) and weight loss > 10%. Blood glucose, plasma insulin, triglycerides and body composition (fat and lean mass) were determined at the Department of Pharmacology and the procedures have been described elsewhere (Jurgens *et al.* 2007).

CRBP-I KO mice

Studies in CRBP-I KO mice have been carried out at Department of Preventive Medicine, Columbia University. Generation of mice lacking CRBP-I has previously

been described (Ghyselinck *et al.* 1999). The mice were backcrossed to the C57BL/6 genetic background for 5 generations prior to experiments described herein. Male mice were placed on a SD (PicoLab® Rodent Diet 20, St. Louis, USA). At the end of the experiment mice were sacrificed, the liver perfused with PBS and tissues excised, flash frozen in liquid nitrogen and stored at -70°C until analysis.

For hypovitaminosis A studies, male CRBP-I KO mice were placed on SD after weaning (PicoLab® Rodent Diet 20, St. Louis, USA). After 9 weeks on SD, the mice were divided into two groups and were continued to be fed either the SD or were started to be fed with a vitamin A deficient (VAD) diet (Research Diets Inc, New Brunswick, USA, no vitamin A/ g diet). The nutritional protocol is represented in Figure 6.

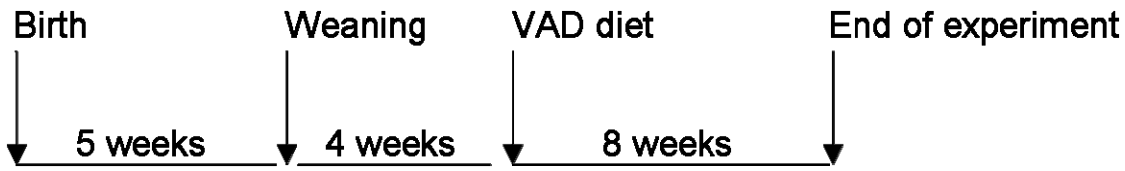

Figure 6 Schematic representation of the nutritional protocol. Mice were fed a standard diet from birth to 9 weeks and then a vitamin A deficient diet for 8 weeks.

All experiments involving mice at the Columbia University were performed with the approval of the Institutional Animal Care and Use Committee at Columbia University.

3.1.2 3T3-L1 cell culture and primary culture of mouse adipocytes

3T3-L1 cells (American tissue culture company, USA) were grown to confluence in basal medium (Dulbecco's modified Eagle's medium, DMEM; Gibco, Carlsbad, USA) with 50 IU/ml penicillin (Sigma, St. Louis, USA), 50 µg/ml streptomycin (Sigma, St. Louis, USA) and 2 mM L-glutamine (Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS, Gemini, New Castle, USA). Two days after the cells reached confluence (referred as day 0) they were induced to differentiate in basal medium containing 10% FBS (Gemini, New Castle, USA), 0.5 µM dexamethasone (Sigma, St. Louis, USA), 0.5 mM methylisobutylxanthine (Sigma, St. Louis, USA) and 10 µg/ml bovine insulin (Sigma, St. Louis, USA) for 2 days, followed by 2 days in basal medium containing 10% FBS and 10 µg/ml insulin. The cells were subsequently refed every other day fresh basal medium supplemented with 10%

FBS and insulin. At day 7, medium was removed and the cells were washed with Phosphate buffered saline (PBS, Gibco, Carlsbad, USA). For the experiment DMEM and 10% FBS containing 5µM ROH (Sigma, St. Louis, USA) or 5µM RA (Sigma, St. Louis, USA) was prepared. 3T3-L1 cells were then incubated for 24 hours with ROH and RA and control medium.

For the primary adipocyte culture epididymal fat pads were removed from 4 nonfasted male WT mice (C57BL/6J, Jackson Laboratory, Maine, USA) which have been maintained on a SD (PicoLab® Rodent Diet 20, St. Louis, USA). The fat pads were washed in PBS (Gibco, Carlsbad, USA) and cut into small pieces (~50 mg). The explants were incubated in DMEM (Gibco, Carlsbad, USA) with 2% fatty acid free bovine serum albumin (BSA, Sigma, St. Louis, USA) as a control and in 2% BSA DMEM supplied with 5µM ROH (Sigma, St. Louis, USA), 5µM RA (Sigma, St. Louis, USA) or 5µM bovine insulin (Sigma, St. Louis, USA) for 6 hours at 37 °C. At the end of the incubation the plates were placed on ice and the medium was removed and stored at -80°C. The remaining tissue pieces were washed twice with ice-cold PBS, shock frozen and saved for protein and RNA isolation. For each animal the assay was performed in quadruplicate.

3.1.3 Human subjects and samples

Lean, overweight and overweight diabetic subjects

A total of 14 adult patients with T2DM (American Diabetes Association criteria) were compared to 28 overweight and 16 lean control subjects which have been recruited within a clinical study at the Department of Clinical Nutrition, German Institute of Human Nutrition Potsdam-Rehbrücke. The diabetes group consisted of 4 males and 10 females. T2DM was defined as fasting blood glucose > 126 mg/dl, 2-hours glucose > 200 mg/dl in a 75-g oral glucose tolerance test (OGTT), or use of insulin or oral hypoglycaemic agents. In control subjects, diabetes was excluded by OGTT. In addition, these individuals did not report any chronic disease. The study protocol was approved by the local ethics committee, and a written informed consent was obtained prior to the study from all participants. Anthropometry was performed as previously described (Spranger *et al.* 2003). Plasma samples were analyzed for glucose, insulin, cholesterol, LDL and HDL cholesterol, triacylglycerides. Creatinine levels were analysed with a Cobas Mira Analyzer (Roche, Mannheim, Germany). The intra-assay coefficients of variation (CV) were: glucose 5.5%; insulin 6%; cholesterol 5.1%; HDL cholesterol 5.4% and

triglycerides 5.1%. Plasma NEFAs (non-esterified fatty acids) were quantified using a colorimetric assay (NEFA, Wako, Neuss, Germany). Inter-assay CV was 4.7%. HbA1c was determined by HPLC as described (Spranger *et al.* 2003).

Subjects suffering from kidney dysfunction

Sera of 18 CKD patients and of 30 patients after RTx were obtained from the Department of Medicine IV, Charité Campus Benjamin Franklin, Berlin, Germany. Kidney function was determined by the estimated glomerular filtration rate (eGFR) which was calculated according to the Modification of Diet in Renal Disease (MDRD) Study formula (Levey *et al.* 1999): the RTx group included patients with eGFR 60 - 11.5 ml/min/1.73 m² and the CKD group included patients with eGFR < 15 ml/min/1.73 m² (National-Kidney-Foundation 2002). CKD patients did undergo HD and serum was obtained immediately predialysis. The mean (± S.E.M.) time duration on dialysis was 14 ± 7 months. The mean dialysis dose (kt/V) was 1.2±0.1. Twenty-five of 30 RTx patients received *Prednisolone*, 15 patients *Cyclosporine* A, 15 patients *Tacrolimus* and 23 RTx patients received *Mycophenolate Mofetil* (MMF). The mean of the period between RTx and sample collection was 2.7 years. Anthropometry and analysis of biochemical serum parameters was performed as previously described (Scholze *et al.* 2007).

Subjects suffering from liver dysfunction

Sera of 63 patients with CLD were obtained from the Department of Hepatology and Gastroenterology, Charité Campus Virchow, Berlin, Germany. Of these patients, 8 were diagnosed with fibrosis METAVIR stage 0 – 1.5, 10 with fibrosis METAVIR stage 2 – 2.5, 9 with METAVIR stage 3, 7 with fibrosis METAVIR stage 4, 10 with hepatocellular cancer (HCC) and 6 with non-alcoholic fatty liver disease (NAFLD). Diagnosis was based on histopathologic, clinical and laboratory findings. Staging was differentiated according to fibrosis: Stage 1 = zone 3 perisinusoidal/pericellular fibrosis, focal or diffuse; stage 2 = focal of diffuse periportal fibrosis together with zone 3 perisinusoidal/pericellular fibrosis; stage 3 = focal and diffuse bridging necrosis together with perisinusoidal/pericellular fibrosis and portal fibrosis; stage 4 = Cirrhosis.

Control group

The sera of 50 healthy subjects were used as control group and were matched for age and body mass index (BMI) with the patient groups. The subjects were recruited as a control group within a clinical study conducted by the Department of Endocrinology, Diabetes and Nutrition, Charité Campus Benjamin Franklin, Berlin, Germany and the Department of Clinical Nutrition, German Institute of Human Nutrition, Potsdam-Rehbrücke, Germany. The inclusion criteria for healthy subjects were no known diagnosis of any kidney or metabolic disease such as obesity/ adiposity, diabetes or hypertension as well as no drug intake. The study protocol was approved by the Ethics Committees of the Charité and the University of Potsdam. Informed consent was obtained from each subject.

After an overnight fast, blood was sampled into EDTA tubes and centrifuged for plasma preparation. Aliquots of plasma were stored at –80 °C until assayed. Body mass index (BMI) was calculated by the formula: weight (kg)/ height (m^2) and eGFR was calculated according to the MDRD study formula (Levey *et al.* 1999).

3.2 Methods

3.2.1 Determination of vitamin A and it esters by reverse phase HPLC

For the separation and quantification of ROH in human serum at the University of Potsdam a gradient reversed-phase high performance liquid chromatography (HPLC) system was used, as previously described (Schweigert 2001). Briefly, 200 µl of ethanol were added to 100 µl plasma (1:1 diluted with water). Afterwards, plasma was extracted twice with n-hexane, stabilized with 0.05% butylated hydroxyluene (BHT), vortexed and centrifuged for 10 min at 1500 g. The supernatants were removed and evaporated under nitrogen and reconstituted in 200 µl isopropanol and injected into the HPLC system (C30 carotenoid column, 5 µm, 250 x 4,6 mm, in line with C18 pre-column, solvent A methanol: water (90:10 v:v, with 0,4 g/l ammonium acetate in water), solvent B methanol:methyl-tert-butyl-ether:water (8:90:2 v:v:v, with 0,1 g/l ammonium acetate in water). The ROH concentration was analysed by calculating the "area under the curve".

Analysis of ROH and RE levels in mouse serum, liver, adipose tissue, pancreas, kidney and heart have been carried out at Department of Preventive Medicine, Columbia University and were determined as previously described (Piantedosi *et al.*

2005). Briefly, serum or tissue sample was mixed with retinylacetate (Sigma, St. Louis, USA) as a standard. Afterwards 4 ml hexane was added and vortexed. The mixture was spinned for 10 minutes at room temperature at 800xg. The upper layer was transferred to a 500 l water and spinned again. The upper layer was dried under liquid nitrogen and then reconstituted in 40 µl Benzene. The subsequent ROH analysis was also performed using a reverse phase HPLC as described before (Vogel *et al.* 2001). The spectra were analysed by a valley-to-valley procedure.

3.2.2 Determination of RBP4 and TTR levels in human serum

Concentrations of RBP4 and TTR were measured by non-commercial enzyme linked immunosorbent assay (ELISA) using polyclonal rabbit anti-human antibodies against RBP4 and TTR (A0040 and A0002, DakoCytomation, Hamburg, Germany) as previously described by this group (Raila *et al.* 2007). For visualization of RBP4 and TTR a second HRP conjugated anti-body (RBP: P0304, DakoCytomation, TTR: 7600-0504, Biotrend, Köln, Germany) was used. TTR and RBP4 standards used in the ELISA were isolated from human serum (N Protein Standard/Standard SL OQIM 13, Dade Behring GmbH, Marburg, Germany), representing the physiological unmodified RBP4 and TTR form. Inter-assay CV´s were 4.2% and 8.1% for RBP4 and TTR, respectively.

Buffers and solutions

10x Phosphate buffered Saline (PBS) pH 6.8: 80 g Sodium chloride, 2 g Potassium chloride, 2 g potassium dihydrogen phosphate, 12.6 g Disodium hydrogen phosphate dihydrate, diluted in 1000 mL deionized water

Carbonate Buffer pH 9.6: A: 10.6 g Sodium carbonate diluted in 1000 mL deionized water. B: 8.4 g Sodium hydrogen carbonate diluted in 1000 mL deionized water. Mix 58.6 mL of A with 141.4 mL of B.

1x PBS / Tween 0.05%: 100 mL 10x PBS, 900 mL Aqua dest., 500 µL Tween20 (Sigma)

Citrate Buffer pH 5.2: A: 21 g Citric Acid Monohydrate (Roth, Karlsruhe, Germany)

diluted in 1000 mL deionized water. B: 35.58g Dinatriumhydrogenphosphatdihydrat (Roth) diluted in 1000 mL deionized water. Mix A and B 1:2.

2.5M Sufuric acid: 13.9 mL Sufuric acid (Roth) diluted in 1000 mL deionized water.

Blocking solution 0.5% BSA/1x PBS: 100 mg BSA diluted in 20 mL 1x PBS.

3.2.3 Western Blotting of proteins in mouse tissues and human serum

Western Blot of mouse serum

To evaluate RBP4 levels in serum of *db/db* mice SDS-PAGE immunoblot analyses was performed. Aliquots of 10µl serum was assayed on 12% reducing SDS-PAGE. The separated proteins were electroblotted onto a polyvinylidene difluoride membrane and Tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% defatted milk was used to block non-specific binding sites on the blot. The membrane was then incubated with anti-RBP4 (A0040, DakoCymation) and washed twice afterwards with TBS 0.3% Tween 20. Then the membrane was incubated with *Envision* labelled polymer HRP conjugated anti-rabbit IgG (K4003, Dako; 1:300 diluted in TBS containing 0.1% Tween 20) for 1 h at room temperature. Antibody binding was visualized using the Luminol reaction (BM Chemiluminescence Blotting Substrate, Roche Diagnostics, Indianapolis, USA).

Buffers and solutions

10x Transfer Buffer: 29.3 g Glycin (Roth, Karlsruhe, Germany), 58.1 g Trisbase (Roth), 3.8 g SDS (Roth) diluted in 800 mL deionized water.

1x Transfer Buffer: 100 mL 10x Transfer Buffer, 700 mL deionized water, 200mL methanol (Roth).

10x TBS: 60.55 g Trisbase (Roth), 87.66 g Sodium chloride (Roth) diluted in 1000 mL deionized water

1x TBS 0.05% Tween 20: 100 mL 10x TBS, 900 mL deionized water, 500 µL Tween 20 (Sigma).

1x TBS 0.10% Tween 20: 100 mL 10x TBS, 900 mL deionized water, 1 mL Tween 20 (Sigma).

1x TBS 0.3% Tween 20: 100 mL 10x TBS, 900 mL deionized water, 3 mL Tween 20 (Sigma).

Blocking solution for one membrane: 10 g milk powder (Roth), 200 mL TBS Tween 0.1%.

Determination of holo- and apo-RBP4 in human serum

Relative amounts of holo-RBP4 and apo-RBP4 in human serum were assessed by using nondenaturating polyacrylamide gel electrophoresis (PAGE) with subsequent immunoblotting analysis. Under these conditions ROH remains bound to RBP4 and due to the higher molecular weight of holo-RBP4 (+ 286Da), two bands may be

detected (Figure 7). The PAGE was performed according to Siegenthaler and Saurat (1987) with slight modifications. The proteins were separated according to their electrophoretic mobilities and subsequently transferred onto a polyvinyl difluoride (PVDF) sheet. Immunoreactive bands were visualized by using rabbit anti-human RBP4 and peroxidase-coupled swine anti-rabbit immunoglobulins (DakoCytomation, Hamburg, Germany). Antibody binding was visualized using the Luminol reaction (BM Chemiluminescence Blotting Substrate, Roche Diagnostics, Mannheim, Germany). Band intensity of both RBP4 isoforms was read with an imager (Bio-Rad, Munich, Germany) and analysed with the Quantity One® software (Bio-Rad). The relative amounts of apo- and holo-RBP4 per lane are expressed as per cent of total intensity of each lane, i.e. since apo- and holo-RBP4 are the only visible bands, the sum of the relative quantities of apo- and holo-RBP4 equals 100% per lane.

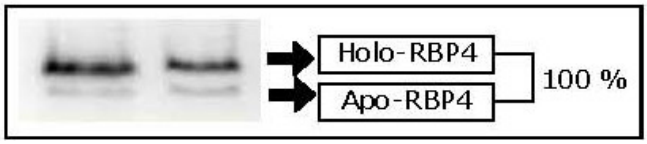

Figure 7 Representative gel after non-denaturating electrophoresis-immunoblotting of apo- and holo-RBP4 in serum of a healthy subject.
Relative amounts of apo- and holo-RBP4 were calculated by comparing the intensity of the apo- band to the holo-RBP4 band of each lane and are displayed as percentage of total intensity per lane.

Buffers and solutions

0.5M Tris/HCl pH 6.8: 30.29 g Trisbase (Roth, Karlsruhe, Germany) diluted in 500mL deionized water (adjust pH with 1M HCL).

1.5M Tris/HCl pH 8.8: 90.87 g Trisbase (Roth) diluted in 500mL deionized water (adjust pH with 1M HCL).

Sample buffer: 1.52 g Trisbase (Roth), 100 mg Bromphenolblau (Sigma), 20 mL Glycerol (Roth) diluted in 1000mL deionized water (adjust pH with 1M HCL).

5x Tris-Glycin: 15.1 g Trisbase (Roth), 72 g Glycin (Roth) diluted in 1000mL deionized water.

Western Blot of 3T3-L1 cells and mouse adipose tissue

The Western Blot analysis of mouse tissues has been carried out at the Department of Medicine, Columbia University. Rabbit polyclonal anti-rat RBP4 antibodies were provided by Dr. R. Piantedosi and were generated as previously described (Muto *et al.* 1972). TTR antibody was purchased from Abnova (Taipeh, Taiwan). Briefly, protein suspensions were analyzed on a 12% SDS-PAGE gradient gel (Bio-Rad,

Hercules, USA), and the protein was transferred to a polyvinylidene difluoride transfer membrane (Millipore, Billerica, USA) by electroblotting. The membrane was blocked in 5% non-fat dry milk (Bio-Rad, Hercules, USA) in Tris-buffered saline, pH 7.4, containing 0.1% Tween-20 (TTBS). The blocked membrane was washed the next morning three times with TTBS and incubated with rabbit antibody for 1 h. The blot was visualized using ECL Western Blotting Detection Reagents (Amersham GE, Piscataway, USA) according to the manufacturer's instructions.

3.2.4 Immunoprecipitation of RBP4 and TTR from human serum and analysis by mass spectrometry

Molecular modifications in the RBP4 and TTR molecule were determined by linear matrix assisted laser desorption / ionization-time of flight - mass spectrometry (MALDI-TOF-MS) after RBP4 immunoprecipitation. For immunoprecipitation 10 µl of serum sample, 10µl Sephadex G-15 (c = 1mg/mL) was incubated with 5 µl amount of polyclonal rabbit anti-human RBP4 (DakoCytomation, Hamburg, Germany) at room temperature for 18 hours, centrifuged at 13.000 rpm for 20 min. After removing the supernatant the protein-antibody complex was washed twice with PBS, pH 7.4. The pellet was resuspended in 10 µl HPLC-grade water. MALDI-TOF-MS was performed as previously described (Gericke *et al.* 2007). Using RBP4 immunoprecipitation it could be shown previously that – apart from RBP4 – TTR is also precipitated) (Schweigert 2007), whereas TTR anti-body does exclusively precipitate TTR. Therefore the TTR molecules which are precipitated by RBP4 anti-body reflect the amount of TTR which is bound to RBP4.

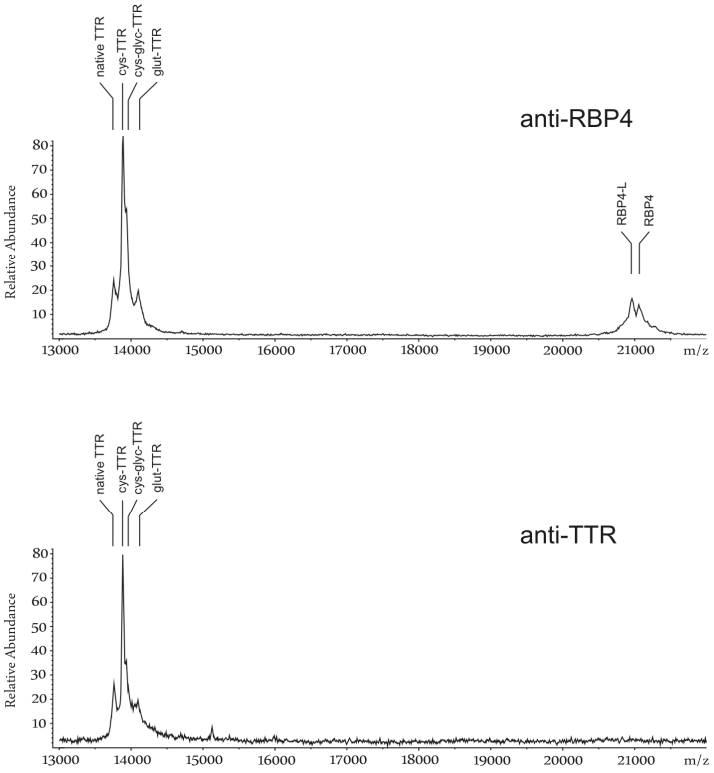

Figure 8 Representative MALDI-TOF mass spectra of the RBP4-TTR complex from plasma using immunoprecipitation with polyclonal retinol-binding protein 4 (anti-RBP4) or polyclonal transthyretin (anti-TTR) antibodies.

For the analysis of the RBP4 spectra and RBP4 truncation the peak heights of non-truncated RBP4 (nRBP4), RBP4 truncated by one leucine molecule (RBP4-L) and by two leucine molecules at the C-terminal end (RBP4-LL) were measured. As the ionization efficiencies of nRBP4, RBP4-L and RBP4-LL are similar, the peaks in the mass spectra reflect the relative amounts of RBP4-L and RBP4 LL of nRBP4 (Jaconi *et al.* 1995). All peak heights were determined in a valley-to-valley procedure and the peak heights of RBP4-L and RBP4-LL are expressed as percentage of the nRBP4. Figure 9 shows representative RBP4 spectra of a healthy control (A) and the spectra of a patient with CKD (B). As the spectra reflect in A, the main peak corresponds to nRBP4. In contrast, in the patient with kidney dysfunction the peak heights of RBP4-L and RBP4-LL are increased compared to nRBP4.

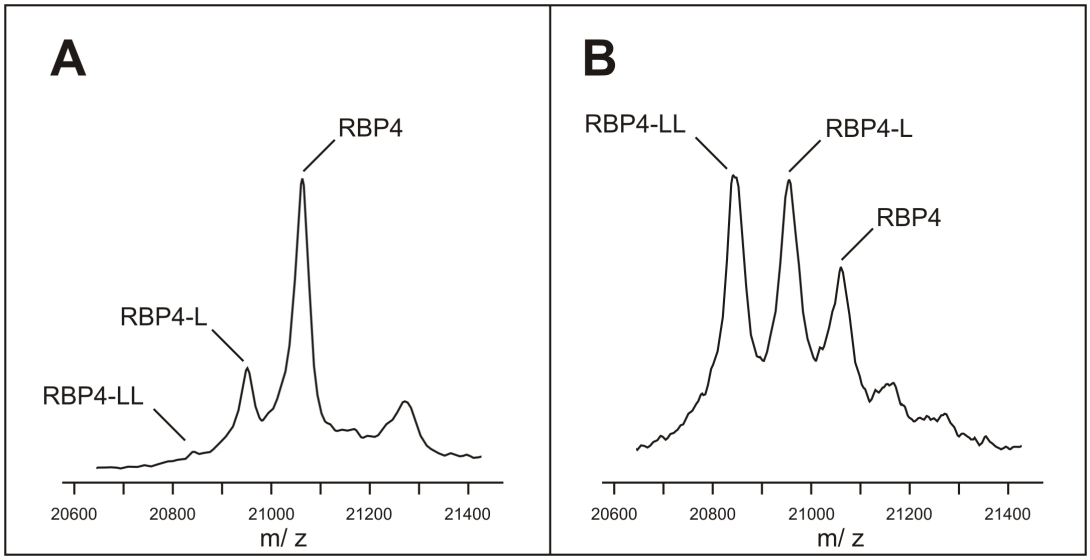

Figure 9 Representative MALDI-TOF mass spectra for RBP4 pattern in control subject (A) and in a patient with kidney dysfunction (B).
RBP4, non-truncated retinol-binding protein 4; RBP4-L, RBP4 truncated at the last C-terminal leucine; RBP4-LL, RBP4 truncated at both C-terminal leucines.

For the analysis of TTR isoforms, which are co-precipitated by RBP4 anti-body and reflect TTR isoforms involved in RBP4 binding, the peaks in the spectra at 14.000 Dalton (molecular weight of TTR) were evaluated. The TTR spectra displays four dominant peaks, which correspond to the native, unmodified TTR form (mass = 13760 Da) and the following three Cys 10 adducts: cys-TTR (mass = 13870 Da), cysglyc-TTR (mass = 13924 Da), and glut-TTR (mass = 14060 Da). Figure 10 shows the typical spectra for TTR and its isoforms. The molecular weight of the TTR isoforms is reproducible with a CV of 0.05%. For reproducibility of relative intensities of TTR isoforms the CVs were 7.6% for cys-TTR and 8.3% for glut-TTR (Henze *et al.* 2008). As the ionization efficiencies of native TTR and its isoforms are also similar, the peaks in the mass spectra reflect the relative amounts of TTR isoforms of native TTR. Therefore peak heights were determined in a valley-to-valley procedure and are expressed as percentage of native TTR.

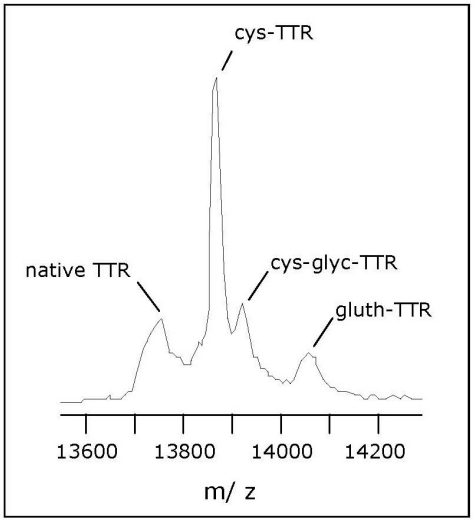

Figure 10 Representative MALDI-TOF mass spectra for TTR pattern in healthy subject.
The TTR spectra displays native TTR (MW = 13760 Da) and three isoforms corresponding to cysteinylated (cys-) TTR (MW = 13870 Da), cysteinglycinylated (cysglyc-) TTR (MW = 13924 Da), and glutathionylated (glut-) TTR (MW = 14060 Da).

3.2.5 Determination of serum parameters in CRBP-I knock-out mice

Blood for determination of lipid and glucose levels was obtained from mice fasted during the day for 6 hours (9 a.m. to 3 p.m.). Serum was obtained after the blood samples had been allowed to clot and subsequent centrifugation. Commercially available kits were used to measure serum levels of free fatty acids (HR Series NEFA-HR, Wako, Richmond, USA), triglycerides (Thermo Scientific, Waltham, USA) and glucose (Accu-Check, Roche Diagnostics, Indianapolis, USA).

3.2.6 Isolation of RNA and protein from mouse tissues

Total RNA from mouse tissues was isolated using RNA-Bee (Tel-Test, Friendswood, USA) according to the manufacturer's protocol. RNA concentration was measured using NanoDrop™ (Thermo Scientific, Wilmington, USA). Samples were stored at -70°C prior to cDNA synthesis and polymerase chain reaction (PCR).

Protein extracts were prepared using 10mL buffer composed of 200µl Tris-Cl (pH 7.5), 100µl EDTA (0.5M), 40µl EGTA (0.5M), 100µl Nonidet P-40 (Sigma, St. Louis,

USA), 1ml NaF (1M), 2 mL sodium pyrophosphate (50mM), proteinase and phosphatase inhibitor cocktails (Roche Diagnostics, Indianapolis, USA). After homogenisation tissue suspension was shaked 1 hour at 4°C and centrifuged at 13.000 rpm for 20min at 4°C. The upper layer (for WAT the middle layer) was transferred into a new tube and stored at -70°C. Western Blot analyses were performed as described on page 25.

3.2.7 Quantitative real-time polymerase chain reaction (Qt-PCR)

Complementary DNA (cDNA) was synthesized from total RNA (3 µg) using the SuperScript-III Reverse Transcriptase (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. The final reaction for the QT-PCR consisted of 0.5 µM of gene specific primer, 1x SYBR Green PCR Master Mix (Fermentas, Maryland, USA) and 10µl diluted cDNA. The reaction was carried out on a Stratagene Cycler. Primer and sequences which have been used are listed in supplemental data (page 106).

3.2.8 Histology

Liver, kidney and WAT were placed in 10% buffered formalin overnight prior to embedding in paraffin. The samples were further processed and paraffin-embedded in a core facility at the Department of Pathology, Columbia University. Sections (5 µM) were applied to Fisherbrand Superfrost/Plus Slides and were stained with haematoxylin and eosin (H&E) using standard procedures. The amount of lipid droplets in the liver was evaluated semi-quantitatively by calculating the relative amount of the intracellular white space of the whole cell. This method has been described before (Kelder *et al.* 2007).

For immunohistochemistry (IHC) at the University of Potsdam polyclonal rabbit anti-human RBP4 and TTR (A0040 and A0002, DakoCytomation) was used 1:400 diluted in 1% BSA in TBS. As a second anti-body swine anti-rabbit IgG (Z0196, Z0196) was used. The third anti-body was a peroxidase-conjugated polyclonal anti-rabbit (Z0113, Z0113) were purchased from DakoCytomation and both were used at a 1:100 dilution in 1% BSA in TBS. The deparaffinized and rehydrated sections were incubated overnight in a humidified chamber at 4°C with the primary anti-RBP4 or anti-TTR antibody. The sections were then exposed to the secondary peroxidase-conjugated antibody for 30 min. Immunoreactivity was visualized using diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 60 mmol/L imidazole buffer (pH 7.1), producing a brown-coloured stain. Counterstaining was

performed with Mayer Hämalaunlösung (Roth). Negative controls, which included the omission of the primary antibodies, had no significant labelling. All incubations except for the primary antibody were performed at room temperature. The sections were examined and photographed with an Olympus BX-50 microscope (Olympus) equipped with a ColorView 12 CCD video camera (SIS). Semi-quantitative analysis was performed as follows: no positive cells observed (-); less than half of cells positive (+); half of more of the cells positive (++); all of the observed cells positive (+++).

Buffer and solutions

10x TBS 0.5M pH 7.6: 60.75 g Trisbase (Roth) diluted in 1000 mL deionized water (adjust pH with 1M HCL).

0.8% Sodium chloride solution: 16 g Sodium chloride (Roth) diluted in 1000 mL deionized water

1x TBS: 100 mL 10x TBS 0.5M pH 7.6, 900 mL 0.8% sodium chloride solution

60mM Imidazol/HCl pH 7.1: 4.09 g Imidazol (Merck) diluted in 1000 mL deionized water (adjust pH with 1M HCL).

1% BSA: 1 mg BSA (Serva, Heidelberg, Germany) and 99 mL 1x TBS.

3.2.9 Plasma Clearance and Uptake of [^{3}H]-Retinoid into Tissues

Postprandial clearance of retinoids by tissues was conducted as previously described (Piantedosi *et al.* 2005). Briefly, a gavage dose was prepared containing [11,12-^{3}H]-ROH (49.3 Ci/mmol; PerkinElmer Life Sciences, Waltham) in peanut oil (concentration: 10^6 cpm/100 µl). After an overnight fast, mice received an oral dose consisting of 100 µl of the oil containing the labelled ROH. Two, 4, 6 and 24 hours after receiving the dose, the mice were sacrificed, and blood and tissues were taken for analysis. The tissues were quickly weighed, immediately flash frozen, and stored at -70 °C until analysis. Serum was obtained after centrifugation of the whole blood at 4 °C. For each time point, four mice for each genotype were used. ^{3}H-Retinoid levels in plasma and tissues were determined as previously described (Piantedosi *et al.* 2005).

3.2.10 Insulin tolerance test in CRBP-I knock-out mice

The insulin tolerance test was performed after the mice had been fasted for 6 hours during the daytime. Blood samples were collected from the tail vein and glucose

levels were measured in duplicates using Accu-Check (Accu-Check, Roche Diagnostics, Indianapolis, USA). Mice received intraperitoneal 0.75 U insulin (Sigma, St. Louis) per g body weight. Blood glucose levels were measured in 15 minute intervals until the insulin tolerance test was finished 90 minutes after the injection of the insulin dose. Five animals for each genotype were used.

3.2.11 *In vitro* assay in HEK293 cells

For the generation of recombinant cDNA for the enzymes MGAT1, MGAT2 and DGAT1 the specific genes were cloned into PCDNA3.0 (Invitrogen, Carlsbad, USA) and amplified in *E. Coli.* The vectors were isolated from *E. Coli* according to the isolation kit used (Quiagen Plasmid Kit, Valencia, USA), the concentration measured and sequences confirmed.

HEK293 cells (American Type Culture Collection, Manassas, USA) were cultivated in 6-well-plates and DMEM (Gibco, Calrsbad, USA) containing 10% FBS (Gemini, New Castle, USA) and 1% penicillin / streptomycin (Sigma, St. Louis, USA). At 90% confluency HEK293 cells were transfected with the transfection reagent Lipofectamine™ 2000 (Invitrogen, Carlsbad, USA) harbouring a plasmid DNA encoding human pcDNA3, pcDNA3-MGAT1, pcDNA3-MGAT2 or pcDNA3-DGAT1 according to the transfection protocol. Briefly, cells in monolayer were washed twice with cold PBS and then cultured in DMEM (Gibco, Carlsbad, USA) with 10% FBS. Then 4 µg vector diluted in Opti-MEM I (Invitrogen, Carlsbad, USA) and lipofectamine (Invitrogen) and incubated for 12 hours. Transfection of MGAT1, MGAT2 and DGAT1 was confirmed by real-time PCR (RNA isolation and PCR procedure described on page 30, primer sequences on page 106). After removal of the transfection medium the ROH containing DMEM was added to the cells and incubated for 18 hours. To test ability of retinyl esterification, cells were incubated with ROH (5 µM) (in DMEM with 1 mg/ml fatty acid free bovine serum albumin (Sigma, St. Louis, USA) and oleic acid (100 µM, Sigma, St. Louis, USA) in the dark. After the incubation period the cells were placed on ice and washed once with ice-cold PBS. The cells were scraped in ice cold PBS from the petri dishes and centrifuged at 240 x g for 10 min at 4 °C. The pellet was shock frozen and later resuspended and homogenized in PBS buffer. Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad) according to the supplier’s instructions. For ROH and RE analysis samples were extracted into

hexane, and the retinyl palmitate content was analyzed by reverse phase HPLC as described above.

3.2.12 Statistical analysis

Results are shown as mean ± standard error of the mean (S.E.M.). Statistic calculations were performed using SPSS 14.0 (SPSS statistical package, SPSS Inc., Chicago, USA). First, Kruskal-Wallis test was applied to evaluate if there were any statistical differences of the investigated parameters among the groups. If there was a significant effect, Mann-Whitney U-rank test was performed to describe differences in proportions between case (patient) and controls (control subject).

For determination of differences between case animal (e.g. knock-out) and control (WT) animal Student's *t*-Test was applied. Non-parametric Spearman rank correlation coefficients were used to test the association between relevant parameters.

Values of $P < 0.05$ (two-tailed) were regarded as statistically significant.

4 Results

4.1 Part 1: Investigations of extracellular retinol-binding proteins

4.1.1 Adipose tissue and liver are involved in obesity whereas the kidney in RBP4 reabsorption

The adipose tissue, kidney and the liver are central organs in ROH metabolism and these organs are affected during obesity. Therefore the effect of obesity in adipose tissue, kidney and liver was investigated in WT mice on SD and on HFD as well as in leptin-deficient *ob/ob* mice on SD. Leptin-deficient *ob/ob* mice show a mutation in the leptin gene and are characterized by an impaired glucose tolerance and hyperinsulinemia. Therefore *ob/ob* mice are a good animal model of obesity and T2DM (Drel *et al.* 2006; Srinivasan and Ramarao 2007). In histology sections the structure of the adipose tissue, the kidney and the liver was investigated as well as the tissue distribution of RBP4 and TTR after immunostaining.

The sections of the epididymal adipose tissue of WT mice on SD, WT mice on HFD and *ob/ob* mice show the typical phenotype of adipocytes: One large lipid droplet displaces the cytoplasm and the nucleus of the adipocyte towards the cell membrane. As depicted in Figure 11, the average adipocyte size is increased in mice on HFD and *ob/ob* mice compared to the cells in WAT of WT mice on SD. Moreover, adipose tissues from mice on HFD and *ob/ob* mice have increased "crowning" around adipocytes which indicates an increased infiltration of macrophages (Figure 11). However, specific cytoplasmatic staining for RBP4 (Figure 11) and TTR (data not shown) was not observed.

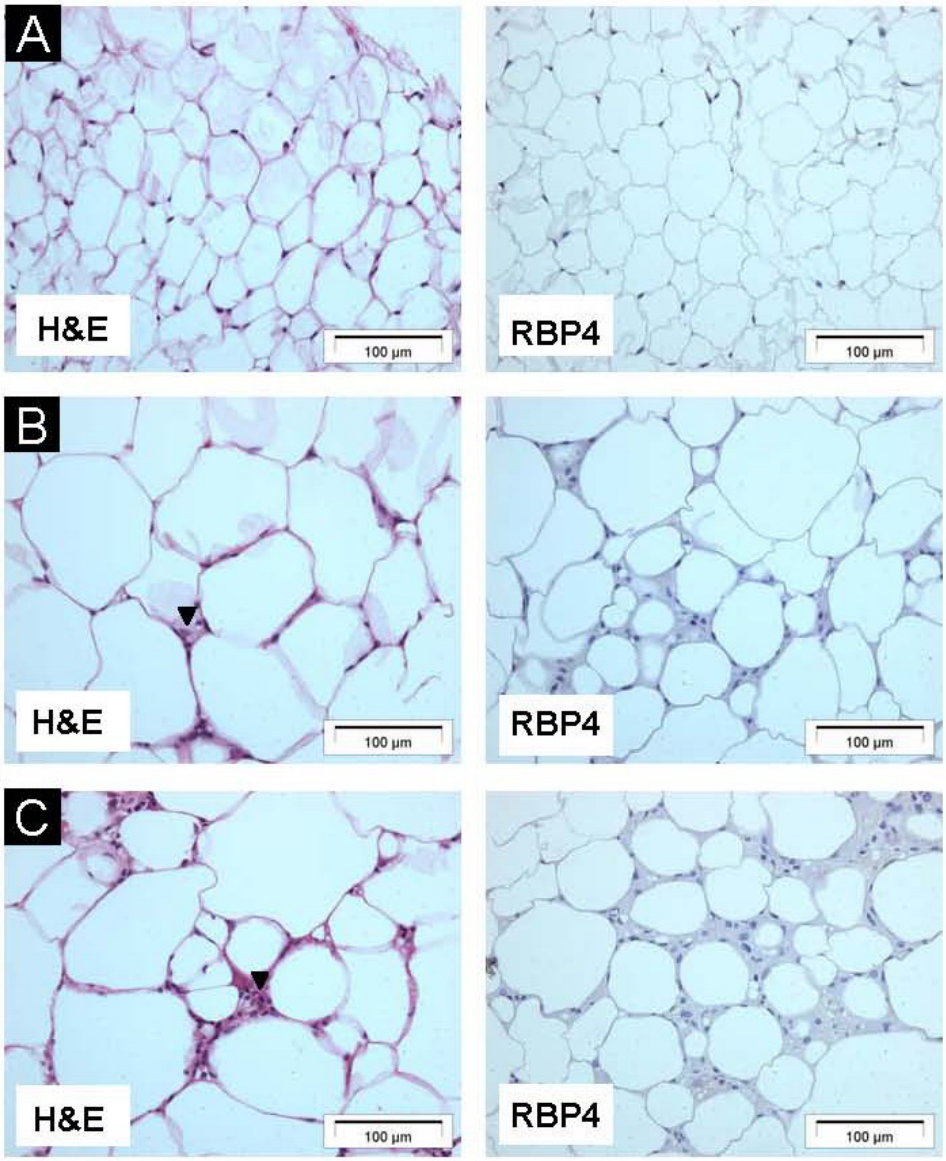

Figure 11 Representative sections of adipose tissue of a wild-type (WT) mouse on standard diet (A), on high fat diet (B) and of an ob/ob mouse (C).
Tissue sections were stained with hematoxylin and eosin (H&E) and RBP4 and TTR antibody. Average adipose cell size is increased in B and C as well as these WAT sections show in increased infiltration of macrophages (arrowheads). There was no immunostaining with RBP4 (right column) and TTR antibody (data not shown).

Besides the adipose tissue as main storage site for ROH, the kidneys play an important role in ROH and RBP4 metabolism due to the active reabsorption of RBP4 and ROH in the tubular system. Moreover, the kidneys are also affected during obesity and diabetes. Figure 12 shows sections of mouse kidneys that were stained with hematoxylin and eosin, anti-RBP4 and anti-TTR. The kidney tissue of WT mice – independent of SD or HFD – was more intact than the tissue of *ob/ob* mice

(Figure 12 C) showing lesions. Moreover, strong RBP4 staining (brown areas, Figure 12) was observed in the tubular cells of the cortex where RBP4 is actively reabsorbed by a megalin-mediated process. The reabsorption was more pronounced in WT mice on SD and *ob/ob* mice (despite lesions in the tissue) compared to WT mice on HFD. TTR staining was also localized in the proximal convoluted tubular cell of the renal cortex but was less strong than the staining for RBP4 (Figure 12).

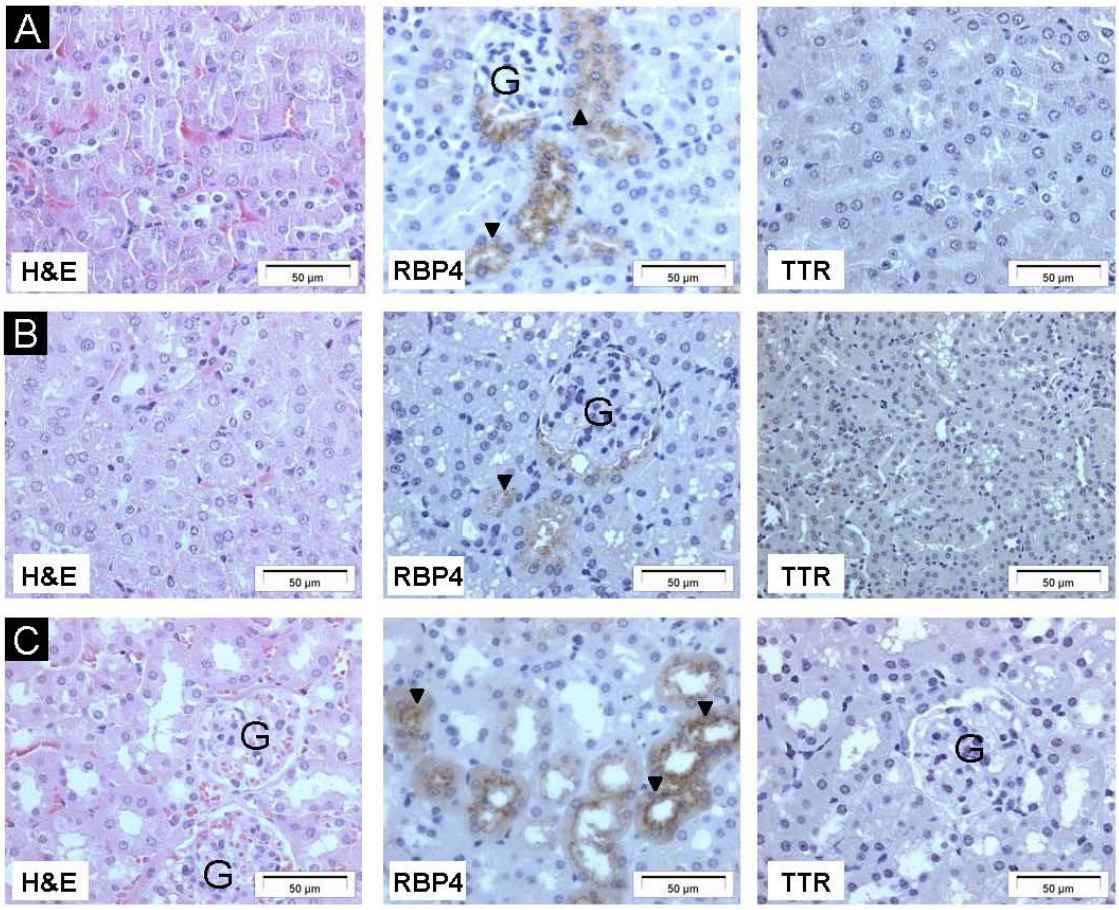

Figure 12 Representative sections of the kidneys of a wild-type (WT) mouse on standard diet (A), on high fat diet (B) and of an ob/ob mouse (C).
Tissue sections were stained with hematoxylin and eosin (H&E) and RBP4 and TTR antibody. Kidney tissue is intact in A and B and RBP4 is localized mainly in the cortex in proximal convoluted tubular cells (arrowheads). Intensity of TTR immunostaining was less than that for RBP4 (right column).

Next, sections of the liver were investigated. The liver is the site of RBP4 synthesis and plays also a role in lipid and glucose metabolism. Moreover, several studies report that obesity is strongly related to an increased risk of liver disease in particular fatty liver disease (Marchesini *et al.* 2008). Therefore liver sections of WT mice on SD and on HFD as well as *ob/ob* mice on SD were investigated. As depicted

in Figure 13 (B, C), livers of WT mice on HFD and *ob/ob* mice show an accumulation of lipid droplets accompanied with a destruction of the liver architecture whereas the livers of WT mice remain intact. In addition, livers of mice on HFD and *ob/ob* mice accumulate lipid droplets (Figure 13). RBP4 and TTR were also localized in the livers of WT mice on SD and HFD as well as of *ob/ob* mice. Specific cytoplasmic staining for RBP4 was observed in liver parenchym cells and the intensity of the staining was mild (arrowheads) in comparison to the intensity in the kidney. Moreover, staining for TTR was observed in parenchym cells but was also mild in WT mice on SD, on HFD and in *ob/ob* mice.

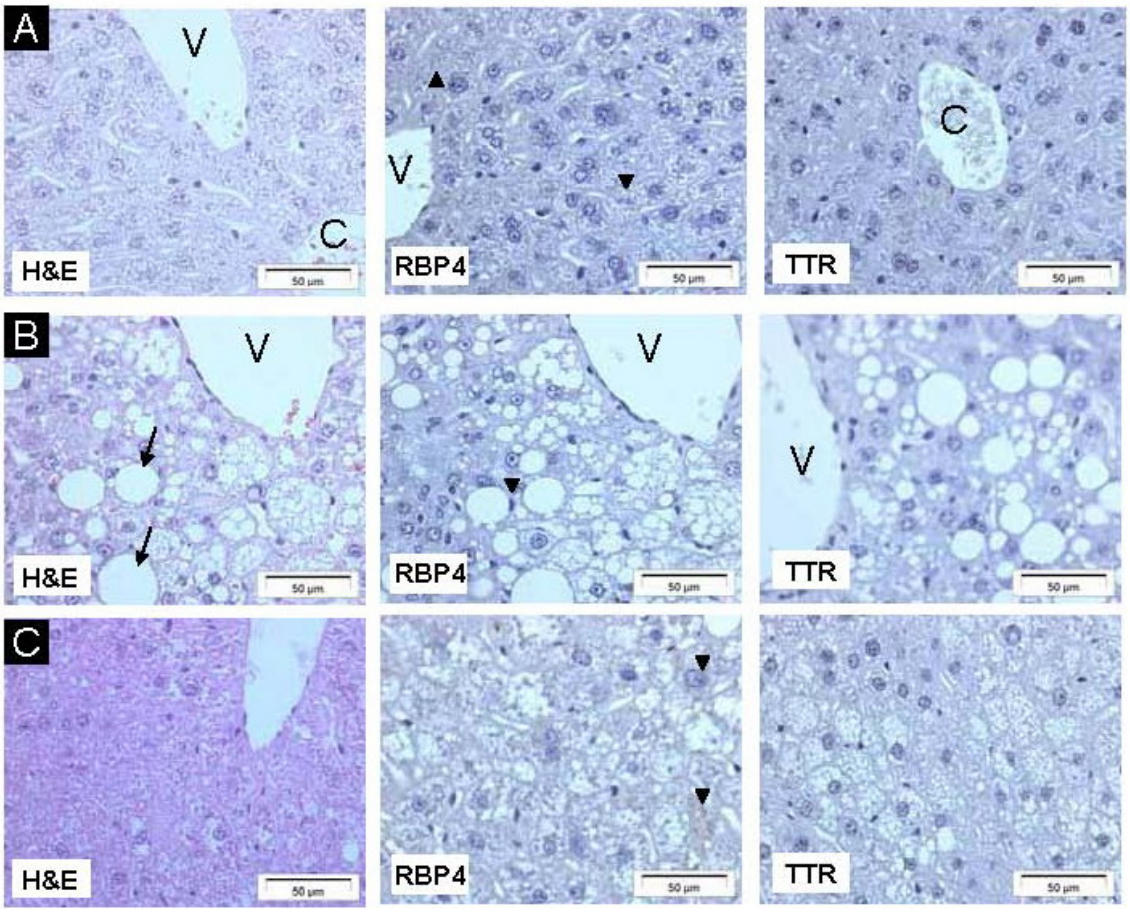

Figure 13 Representative sections of the liver of a wild-type (WT) mouse on standard diet (A), on high fat diet (B) and of an ob/ob mouse (C).
Tissue sections were stained with hematoxylin and eosin (H&E) and RBP4 and TTR antibody. Liver tissue is intact in A compared to tissues of B and C which are characterized by the accumulation of lipid droplets (arrows). RBP4 is localized in small amounts (arrowheads); intensity of TTR immunostaining was mild (right column). V, venules; C, capillaries.

Table 1 summarizes the immunoreactivity of RBP4 and TTR in the investigated sections of the WAT, kidney and liver. The strongest staining was observed for RBP4 in the kidneys, whereas RBP4 staining intensity in the liver was less strong.

In general, TTR staining was not very strong. There was no RBP4 and TTR staining observed in the adipose tissue.

Table 1 Immunoreactivity of RBP4 and TTR in WAT, the kidney and liver of WT mice on standard (SD) and high fat diet (HFD) and of ob/ob mice on SD.

Protein	SD	HFD	*ob/ob*
WAT RBP4	-	-	-
WAT TTR	-	-	-
Kidney RBP4	++	++	+++
Kidney TTR	+	+	+
Liver RBP4	++	++	+
Liver TTR	-	-	+

-, no positive cells observed; +, some cells were positive; ++, 20 per cent of cells were positive; +++, 50 per cent of cells were positive.

In addition to histology the expression levels of RBP4 and TTR were analyzed in WAT, kidneys and liver. RBP4 expression was detected in WAT, the kidney and the liver. The highest expression levels of RBP4 were observed in the liver compared to the WAT and the kidneys. Table 2 shows the relative expression levels in mice on HFD and *ob/ob* mice in proportion to the levels in mice on SD. TTR is expressed in the kidney and the liver but also in the WAT of WT mice as well as in WT mice on HFD and *ob/ob* mice. There were no differences among the groups which is probably due to the small sample size of n = 3).

Table 2 Expression levels of RBP and TTR in WT mice on high fat diet (HFD) and in ob/ob mice in WAT, kidneys and liver.

Gene	HFD	ob/ob
WAT RBP4	0.43 ± 0.13	0.17 ± 0.01
WAT TTR	52.85 ± 22.26	5.42 ± 2.48
Kidney RBP4	10.40 ± 9.33	8.08 ± 6.41
Kidney TTR	3.05 ± 1.92	1.28 ± 0.41
Liver RBP4	0.85 ± 0.38	1.09 ± 0.78
Liver TTR	0.71 ± 0.11	0.96 ± 0.14

Gene expression is presented as relative expression level and as proportion of expression level in WT mice on SD (mean ± S.E.M., n=3 each group). There were no significant differences between the groups.

4.1.2 Adipocytes secrete RBP4 and TTR

Several studies reported that the increase in serum RBP4 levels during obesity and T2DM is the result of an increased RBP4 secretion from WAT (Janke *et al.* 2006; Yao-Borengasser *et al.* 2007). The regulation and secretion of RBP4 from the liver has been extensively studied, but the regulation of RBP4 expression and secretion in adipose tissue remains unclear. Furthermore, it is not known whether ROH is secreted bound to RBP4 and TTR from adipocytes. In fact, TTR expression and secretion from adipocytes has not been studied yet. This is surprising since adipose tissue is an important storage site for retinoids and plays an important role in ROH metabolism. Thus, Western Blotting was used to confirm previously published results on RBP4 protein levels in adipose tissue indicating that RBP4 is present in WAT of WT mice, *ob/ob* and *db/db* mice. Figure 14 shows that the RBP4 band of adipose tissue from *ob/ob* mice is more intense compared to the band in WT mice (relative to total protein). Moreover, in *db/db* mice RBP4 levels in WAT were slightly increased compared to WT mice.

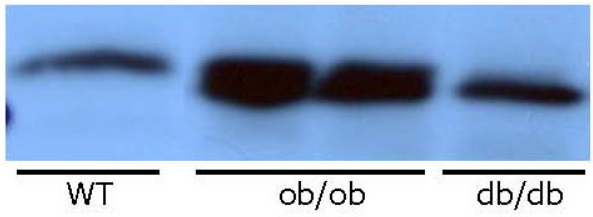

Figure 14 RBP4 in adipose tissue of wild-type (WT), ob/ob and db/db mice. Representative Western blots are shown with 40 µg of total WAT protein per lane.

To further investigate the secretion of RBP4 and TTR, two cell culture models were used: 3T3-L1 cells and primary adipocytes.

First, 3T3-L1 cells were differentiated and tested whether these cells are a useful model for the study of the tissue-specific regulation of RBP4 and TTR expression and secretion. Although 3T3-L1 cells express RBP4 and TTR ($2^{-\Delta ct}$ 0.01 ± 0.01 and 0.02 ± 0.01, respectively), the mRNA levels as well as the protein levels of RBP4 and TTR secreted into medium were very low. Therefore, the data obtained from 3T3-L1 cells was not further analyzed.

Second, primary adipocytes from epididymal WAT of WT mice were cultivated as described above (page 20). RBP4 and TTR expression levels were detectable in high amounts in primary adipocytes ($2^{-\Delta ct}$ 393.58 ± 10.71 and 10.64 ± 3.98, respectively). Therefore, primary adipocytes were used to investigate alterations in expression and secretion of RBP4 and TTR caused by the incubation with ROH, RA and insulin for 6 hours. After the incubation period RBP4 and TTR expression was analysed. RBP4 expression was not altered after treatment with ROH, RA and insulin (Table 3) as well as TTR expression was not altered after 6 hours of incubation.

Table 3 Relative expression levels of RBP4 and TTR in primary adipocytes of wild-type mice after incubation with retinol (ROH), retinoic acid (RA) and insulin.

Treatment	RBP4	TTR
-	1.43 ± 0.32	0.06 ± 0.04
ROH	1.27 ± 0.28	0.14 ± 0.07
RA	1.31 ± 0.20	0.25 ± 0.18
Insulin	1.31 ± 0.39	0.06 ± 0.02

Values (relative expression levels) are means ± S.E.M. (n=5 for each genotype). There was no significant difference between the different treatments.

The secretion of RBP4 and TTR into medium after treatment was evaluated by Western Blotting. RBP4 secretion into medium remained unchanged after treatment (data not shown). Interestingly, it could be shown here that primary mouse adipocytes secrete TTR into medium and that there was a tendency of an increased TTR secretion into medium after treatment with ROH (Figure 15).

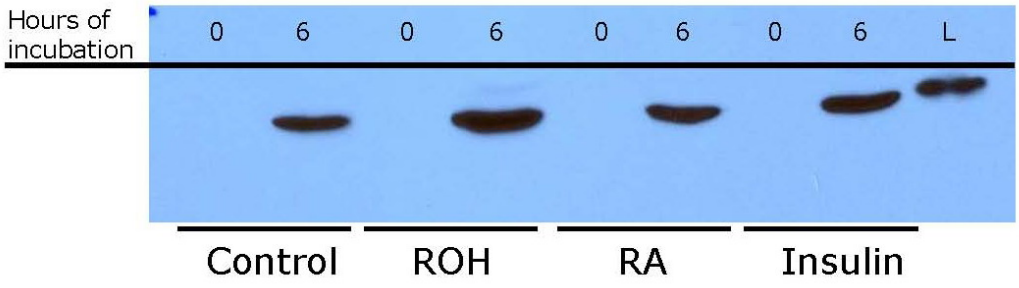

Figure 15 Primary adipocytes secrete TTR into medium.
The TTR Western Blot shows medium before incubation with primary adipocytes and 6 hours after incubation with retinol (ROH), retinoic acid (RA) and insulin. Liver (L) extract was used as positive control.

4.1.3 Diabetic *db/db* mice have increased RBP4 serum and also creatinine levels

The *db/db* mouse strain exhibits a mutation in the leptin receptor gene leading to hyperglycaemia and insulin resistance (Srinivasan and Ramarao 2007). In detail, the *db/db* mice suffer from an obesity syndrome associated with early hyperinsulinemia followed by hyperphagia, severe insulin resistance and either a transient (mild) or sustained (severe) hyperglycaemia (Leiter *et al.* 1983). Therefore this animal model was used to study the impact of obesity and T2DM on RBP4 serum levels. The aim was to evaluate RBP4 serum levels in diabetic mice maintained on SD and HFD and the relation to kidney function.

For the investigation of RBP4 serum levels in *db/db* mice (n=16) and WT mice (n=12) were maintained on SD or HFD beginning at week 5 for 22 weeks. However, at the end of the intervention *db/db* mice on SD were diabetic as evaluated by glucose tolerance test and euglycaemic-hyperinsulinaemic clamp technique. Body weight and blood glucose levels were increased in *db/db* mice on SD and HFD compared to WT mice on SD and HFD ($P<0.05$, all), respectively (Figure 16 A, B). In addition, relative RBP4 levels in relation to a standard serum were increased in *db/db* mice on SD compared to WT mice on SD ($P<0.05$) and in *db/db* mice (SD and HFD) compared to WT mice on HFD ($P<0.05$). Since the kidneys are often affected during diabetes and may strongly elevate RBP4 levels during their dysfunction, serum creatinine – as a marker of kidney function – was evaluated in these mice. Interestingly, as shown in Figure 16 serum creatinine levels were also increased in both *db/db* groups compared to the WT mice on HFD ($P<0.05$, both). The differences in creatinine levels in *db/db* mice compared to WT on SD was not significant probably due to the small sample size[5]: P-values for creatinine levels in *db/db* mice on HFD and on SD compared to WT mice on SD were $P=0.08$ and 0.06, respectively.

[5] In this group (WT mice on SD) creatinine levels were detemined in a subgroup (n=2) of samples due to the obligatory large amount of serum sample which is needed for the assay. This explains the lack of differences between WT mice on SD and *db/db* mice in the creatinine levels.

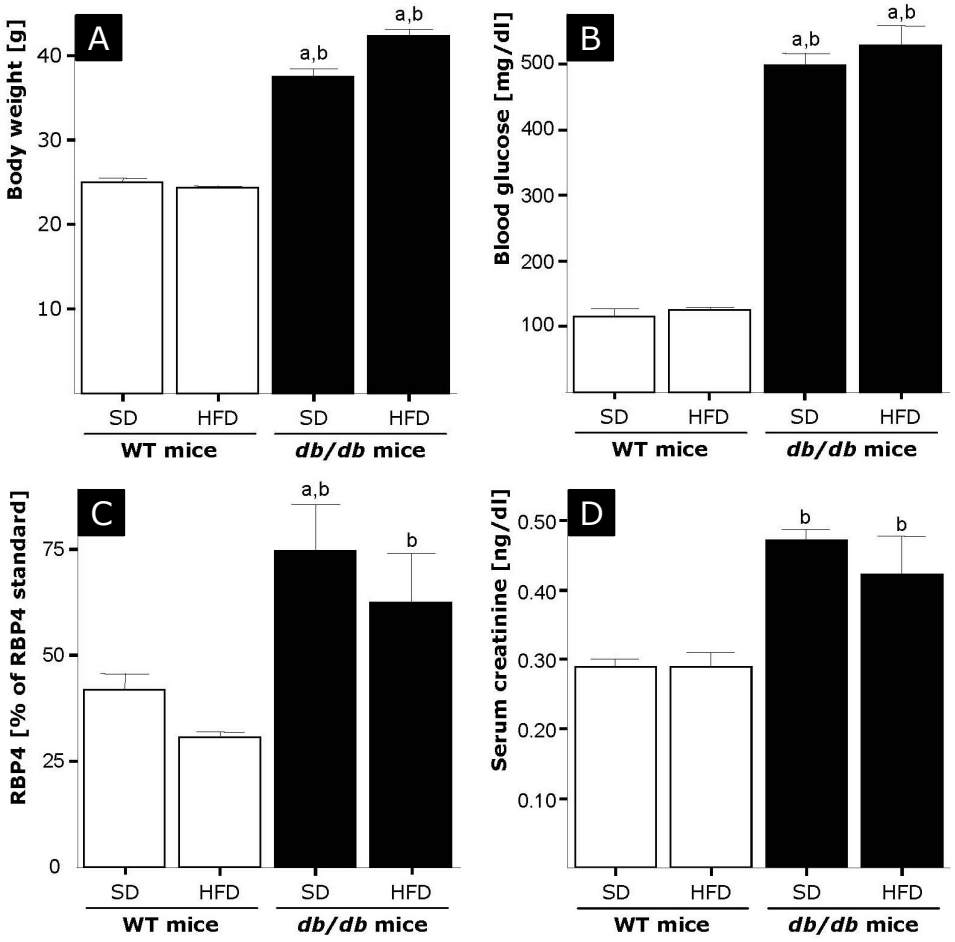

Figure 16 Effect of diabetes on RBP4 serum levels in db/db mice.
Body weight (A), blood glucose levels (B) are increased in db/db mice compared to wild-type (WT) mice. a, significant different to WT mice on standard diet (SD), $P < 0.05$; b, significant different to WT mice on high-fat diet (HFD), $P < 0.05$. RBP4 is displayed as percentage of the band intensity of a standard serum.

RBP4 levels were correlated with body weight ($r = 0.63$, $P < 0.01$), relative body fat amount ($r = 0.69$, $P < 0.01$), blood glucose ($r = 0.42$, $P < 0.05$), insulin levels ($r = 0.50$, $P < 0.05$), levels of free fatty acids ($r = 0.46$, $P < 0.05$) and serum creatinine ($r = 0.70$, $P < 0.05$) levels in the whole study group. Moreover, there were also positive correlations of blood glucose and body weight ($r = 0.73$, $P < 0.01$), relative amount of body fat ($r = 0.84$, $P < 0.01$) and creatinine levels ($r = 0.67$, $P < 0.05$) in the whole study group.

4.1.4 RBP4 and TTR are not altered in overweight and overweight diabetic human subjects

To further expand the data on RBP4 and the possible influence of kidney function evaluated in diabetic *db/db* mice a study in diabetic human subjects was performed. Measurements of RBP4 and TTR levels as well as analysis of RBP4 and TTR isoforms were carried out in the sera of 28 overweight and 14 overweight T2DM subjects. Sera of 16 lean subjects were used as a control group.

Anthropometrical and clinical characteristics of lean controls, overweight subjects and overweight subjects with T2DM are shown in Table 4. Overweight T2DM subjects were older ($P < 0.05$) compared with overweight but not lean controls. BMI and waist-to-hip ratio (WHR) were higher in both overweight groups ($P < 0.01$). Compared to lean controls, parameters characteristic for an impaired glucose metabolism, such as fasting plasma glucose, fasting insulin, HOMA-IR as well as concentrations of HbA1c and triglycerides were increased ($P < 0.001$, all) in overweight subjects with T2DM. Levels of insulin, HOMA-IR, cholesterol, and LDL-cholesterol were also increased ($P < 0.01$, all) in plasma of overweight controls compared to the lean controls. Between overweight controls and overweight T2DM subjects, differences were found in plasma glucose, HbA1c ($P < 0.001$, both), HOMA-IR ($P < 0.01$), and triglyceride levels ($P < 0.05$), but the ratio of the incremental response of insulin to glucose at 30 minutes of the OGTT (ΔI30/ΔG30) was not significantly different. With regard to kidney function, no significant differences in the concentration of plasma creatinine ($P = 0.73$; Kruskal-Wallis test) and eGFR ($P = 0.98$) estimated by the MDRD Study Group formula were evident among the groups.

Table 4 Anthropometric and biochemical characteristics of lean controls, overweight controls, and overweight T2DM patients.

	Lean controls	Overweight controls	Overweight T2DM	P-value [1]
N (% m/ % f)	16 (23/87)	28 (36/64)	14 (29/71)	
Age (years)	51.6 ± 3.6	50.3 ± 1.8	59.9 ± 2.5	0.027
BMI (kg/m²)	21.7 ± 0.6	29.6 ± 0.9	31.5 ± 1.9	0.001
WHR	0.82 ± 0.02	0.89 ± 0.02	0.93 ± 0.02	0.005
SBP (mm Hg)	116 ± 4	124 ± 4	133 ± 3	0.009
DBP (mm Hg)	74 ± 2	80 ± 2	78 ± 2	0.028
Glucose (mmol/L)	4.74 ± 0.13	4.79 ± 0.09	6.53 ± 0.24	0.001
Insulin (µU/l)	5.58 ± 1.22	9.16 ± 0.97	13.3 ± 2.30	0.001
HbA1c (%)	5.40 ± 0.28	5.37 ± 0.10	6.08 ± 0.20	0.001
HOMA-IR	1.21 ± 0.28	1.99 ± 0.24	6.08 ± 0.66	0.001
ln ($\Delta I_{30}/\Delta G_{30}$) [2]	4.11 ± 0.12	4.53 ± 0.12	4.25 ± 0.66	n.d.
Total cholesterol (mg/dl)	5.06 ± 0.14	5.78 ± 0.18	5.54 ± 0.34	0.048
LDL-cholesterol (mg/dl)	3.03 ± 0.15	3.74 ± 0.16	3.27 ± 0.25	0.023
HDL-cholesterol (mg/dl)	1.56 ± 0.09	1.33 ± 0.05	1.30 ± 0.07	n.d.
Triglyceride (mg/dl)	1.00 ± 0.08	1.54 ± 0.18	2.28 ± 0.39	0.001
eGFR (mL/min per 1.73 m²)	78.5 ± 2.4	80.8 ± 2.8	77.7 ± 4.5	n.d.

Data are expressed as mean ± S.E.M. n.d., not different; m, male; f, female; BMI, body mass index; WHR, waist-to-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycosylated haemoglobin; HOMA-IR, homeostasis model assessment of β-cell function and insulin resistance; eGFR, estimated glomerular filtration rate. [1] Kruskal-Wallis test. [2] estimated Δinsulin 30 to Δglucose 30 = insulin 30 - insulin 0 / glucose 30 – glucose 0).

With regard to the components of the vitamin A transport complex, RBP4 serum levels did not differ among lean controls, obese controls, and obese subjects with T2DM (Table 5). The ratio of holo- and apo-RBP4 which indicates the binding of ROH to RBP4 did not vary among the groups. In addition, serum TTR concentrations did also not differ between the groups (Table 5).

Besides the above mentioned quantitative analysis of RBP4 and TTR serum levels, both proteins may also be modified in their molecular structure under certain pathophysiological conditions. To that date kidney dysfunction is known to be such an influencing factor. Therefore the RBP4 and TTR mass spectra were analysed for modifications (i.e. isoforms) after RBP4 immunoprecipitation. The analysis of the RBP4 spectra showed three peaks: the RBP4 peak (mass = 21.065 Da), the RBP4-L peak (mass = 20.950 Da) and the RBP4-LL peak (mass = 20.837 Da) reflecting non-truncated RBP4, RBP4 truncated at the C-terminal end by one leucine and RBP4 truncated at the C-terminal end by two leucines, respectively. The intensity of the non-truncated RBP4 (nRBP4) was set to 100% and RBP4-L and RBP4-LL are expressed as per cent of nRBP4. There were no differences in RBP4 isoforms among controls, overweight controls and overweight T2DM patients (Table 5).

The RBP4 antibody which was used for immunoprecipitation does also precipitate TTR from serum. Therefore the obtained TTR spectra reflect the TTR which is bound to RBP4. The TTR spectra displayed four dominant peaks, which correspond to its native, unmodified form (mass = 13760 Da) and the following three adducts at the position 10 of the amino acid cysteine (TTR isoforms): cys-TTR (mass = 13870 Da), cysglyc-TTR (mass = 13924 Da), and glut-TTR (mass = 14060 Da). In all groups cys-TTR was the most abundant form compared to the peak intensity of cysglyc-TTR, glut-TTR, and native TTR. Moreover, no differences in TTR isoforms were detected between lean and overweight controls and overweight T2DM subjects (Table 5).

Table 5 Parameters of the RBP4-TTR complex in plasma of lean controls, overweight controls, and overweight T2DM patients.

	Lean Controls (n=16)	**Overweight controls (n=28)**	**Overweight T2DM (n=14)**	**P-value [1]**
RBP4 (µmol/L)	1.95 ± 0.09	2.12 ± 0.15	1.89 ± 0.14	n.d.
TTR (µmol/L)	6.66 ± 0.58	6.67 ± 0.53	6.11 ± 0.62	n.d.
Holo-RBP4 (%)[2]	84.1 ± 1.7	85.44 ± 0.67	84.2 ± 88.4	n.d.
Apo-RBP4 (%)[2]	17.9 ± 2.4	13.5 ± 1.1	15.5 ± 1.4	n.d.
RBP4-L (% of non-truncated RBP4) [3]	29.4 ± 3.1	24.0 ± 3.7	31.7 ± 6.6	0.04 [4]
RBP4-LL (% of non-truncated RBP4) [3]	0.7 ± 0.5	0.6 ± 0.3	1.6 ± 0.7	n.d.
Cys-TTR (% of native TTR)	474 ± 47	536 ± 35	617 ± 77	n.d.
Cysglyc-TTR (% of native TTR)	53 ± 9	51 ± 7	56 ± 10	n.d.
Glut-TTR (% of native TTR)	91 ± 10	96 ± 6	94 ± 10	n.d.

Data are expressed as mean ± S.E.M. n.d., not different; T2DM, type 2 diabetes; RBP4, retinol-binding protein 4; TTR, Transthyretin; holo-RBP4 / apo-RBP4, RBP4 bound / not bound to ROH; RBP4-L / RBP4-LL, RBP4 truncated at the C-terminal end by one / two leucine molecules; cys-TTR, S-cysteinylated transthyretin; cys-glyc-TTR, S-cysteinglycinylated transthyretin; glut-TTR, S-glutathionylated transthyretin. The RBP4/TTR index is the molar ratio of plasma RBP4 to plasma TTR. [1] Kruskal-Wallis test; [2] Holo- and apo-RBP4 were calculated by comparing the intensity of the apo- band to the holo-RBP4 band of each lane (sample) after non-denaturating electrophoresis-immunoblotting and are displayed as percentage of total intensity per lane which has been set 100%. [3] The intensity of the non-truncated RBP4 peak in the mass spectra was set 100% and the intensities of RBP4-L and RBP4-LL were expressed in % of non-truncated RBP4. [4] P = 0.04 for lean and overweight controls.

4.1.5 RBP4 and TTR levels and isoforms are altered in human subjects with kidney dysfunction

Since there was no difference in RBP4 and TTR levels in overweight and overweight T2DM patients, RBP4 and TTR levels and isoforms were investigated in patients with kidney dysfunction. Diminished renal function is the only known disease in which RBP4 serum levels are chronically elevated. Decreased kidney function and ESRF is

often related to T2DM and in chapter 4.1.3 it has been shown that there is a strong correlation among RBP4 and creatinine levels in diabetic mice (page 42). Therefore the aim of this cross-sectional study was to characterize RBP4 and TTR levels as well as isoforms in patients with kidney dysfunction. The investigated subjects suffered from CKD (stage 5) and did undergo haemodialysis or have been undergoing renal transplantation (RTx).

Anthropometric and biochemical characteristics of controls, CKD and RTx patients are shown in Table 6. Systolic blood pressure, triglycerides, haemoglobin, hematocrit and serum CRP were elevated in both patient groups compared to controls ($P < 0.001$, both). Blood glucose levels were increased in RTx compared to controls ($P < 0.05$), but were not different from the CKD group. Highest eGFR was observed in controls whereas in CKD and RTx the rate was decreased ($P < 0.001$, both). In the CKD group and the RTx group 3 and 5 patients, respectively, were suffering from T2DM.

Table 6 Anthropometric and biochemical characteristics of controls, patients with chronic kidney disease (CKD) and patients after renal transplantation (RTx).

Variable	Controls	CKD	RTx	P-Value [1]
N (% m / % f)	35 (63 / 37)	18 (61 / 39)	30 (67 / 33)	
Age (years)	58 ± 1	57 ± 3	56 ± 2	n.d.
BMI (kg/ m²)	26.2 ± 1.9	24.9 ± 1.0	26.0 ± 1.1	n.d.
SBP (mm Hg)	121.4 ± 2.2	145.8 ± 7.0	147.4 ± 5.9	< 0.001
DBP (mm Hg)	75.6 ± 1.5	81.7 ± 4.8	81.3 ± 3.5	n.d.
Glucose (mmol/L)	4.7 ± 0.08	6.3 ± 1.2	6.3 ± 0.4	< 0.001
Triglycerides (mmol/ L)	1.1 ± 0.1	1.9 ± 0.2	2.3 ± 0.5	< 0.001
CRP (nmol/ L)	1.8 ± 1.3	875.8 ± 212.0	437.7 ± 113.3 [2]	< 0.001
eGFR (mL/ min/ 1.73 m²)	85.6 ± 2.3	9.8 ± 1.5	28.5 ± 4.5 [2]	< 0.001
Hb (g/L)	141.7 ± 2.3	113.0 ± 6.6	111.5 ± 4.8	< 0.001
Ht	0.42 ± 0.00	0.33 ± 0.01	0.29 ± 0.02	< 0.001
Diagnosis of Diabetes	0	3	5	

Data are expressed as mean ± S.E.M. n.d., not different; m, male; f, female; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; Hb, haemoglobin; Ht, hematocrit; n.d., not different. [1] Kruskal-Wallis-Test; [2] significant different between RTx and CKD (p < 0.05).

With regard to the components of the vitamin A transport complex, serum RBP4 levels were strongly elevated in CKD and RTx patients compared to controls (Table 7). Thereby the elevation of RBP4 was more pronounced in RTx compared to CKD patients ($P < 0.01$). ROH was elevated in RTx compared to controls and CKD ($P < 0.001$). TTR was decreased in CKD compared to controls and RTx ($P < 0.01$).

Table 7 Parameters of the RBP4-TTR-complex in serum of controls, CKD and RTx patients.

Variable	Controls (n = 35)	CKD (n = 18)	RTx (n = 30)	P-Value [1]
ROH (µmol/ L)	1.54 ± 0.06	1.62 ± 0.27	2.63 ± 0.27 [4]	< 0.001
RBP4 (µmol/ L)	2.18 ± 0.08	3.87 ± 0.39	7.19 ± 0.86 [4]	< 0.001
TTR (µmol/ L)	6.22 ± 0.24	4.96 ± 0.89	8.38 ± 0.93 [4]	< 0.05
Holo-RBP4 (%) [2]	86.90 ± 2.34	59.38 ± 3.89	70.25 ± 2.94 [4]	< 0.001
Apo-RBP4 (%) [2]	13.09 ± 2.34	37.80 ± 2.86	29.75 ± 2.94 [4]	< 0.001
RBP4-L (% of non-truncated RBP4) [3]	57.57 ± 9.09	113.82 ± 34.29	166.44 ± 25.35	< 0.05
RBP4-LL (%of non-truncated RBP4) [3]	3.57 ± 1.64	112.76 ± 58.87	92.96 ± 23.21	< 0.001
Cys-TTR (% of native TTR)	235.90 ± 16.75	368.48 ± 60.14	742.71 ± 88.43 [4]	< 0.001
Cysglyc-TTR (% of native TTR)	29.52 ± 3.62	37.65 ± 11.99	63.86 ± 10.64	< 0.05
Glut-TTR (% of native TTR)	44.72 ± 3.2	84.12 ± 8.66	107.82 ± 13.34	< 0.001

Data are expressed as mean ± S.E.M. n.d., not different; CKD, patients with chronic kidney disease, stage 5; RTx, patients after renal transplantation; ROH, retinol; RBP4, retinol-binding protein 4; TTR, transthyretin; holo-RBP4 / apo-RBP4, RBP4 bound / not bound to ROH; RBP4-L / RBP4-LL, RBP4 truncated at the C-terminal end by one / two leucine molecules. n. d., not different. [1] Kruskal-Wallis-Test [2] Holo- and apo-RBP4 were calculated by comparing the intensity of the apo- band to the holo-RBP4 band of each lane (sample) after non-denaturating electrophoresis-immunoblotting and are displayed as percentage of total intensity per lane which has been set 100%. [3] The intensity of the non-truncated (native) RBP4 peak in the mass spectra was set 100% and the intensities of RBP4-L and RBP4-LL are expressed in % of non-truncated RBP4. [4] significant different between RTx and CKD.

In addition, analysis of band area under the curve after non-denaturating PAGE immunoblotting was used to quantify the relative amount of apo- and holo-RBP4 (Figure 17). The ratio of apo- to holo-RBP4 is used to assess the degree of RBP4 saturation with ROH. The relative amount of holo-RBP4 was decreased in CKD compared to controls and RTx ($P < 0.01$, both); vice versa apo-RBP4 was detected in higher intensities in CKD and RTx compared to controls ($P < 0.01$, both, Table 7). In RTx apo-RBP4 was decreased and holo-RBP4 was increased compared to CKD ($P < 0.05$).

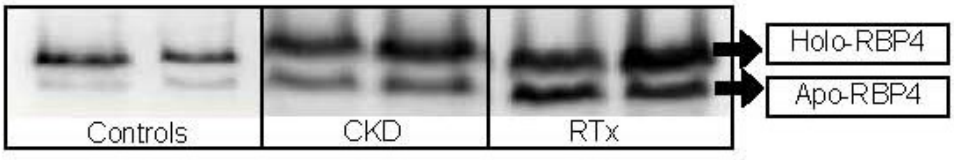

Figure 17 Representative gel after non-denaturating electrophoresis-immunoblotting of apo- and holo-RBP4 in serum of controls and patients with chronic kidney disease (CKD) and patients after renal transplantation (RTx).
Relative amounts were calculated by comparing the intensity of the apo- band to the holo-RBP4 band of each lane and are displayed in Table 7 as percentage of total intensity per lane.

To investigate RBP4 isoforms, RBP4 immunoprecipitation and subsequent MALDI-TOF-MS analysis was performed. Different peak patterns of the detected molecular masses of RBP4 (non-truncated), RBP4-L and RBP4-LL in CKD and RTx patients compared to controls were observed (exemplified in Figure 18). As depicted in Table 7, significantly higher relative amounts of RBP4-LL were detected in CKD and RTx compared to controls ($P < 0.01$, both). In addition, RBP4-L was increased in RTx compared to controls ($P < 0.001$). RBP4-L and RBP4-LL were not different among HD and RTx patients.

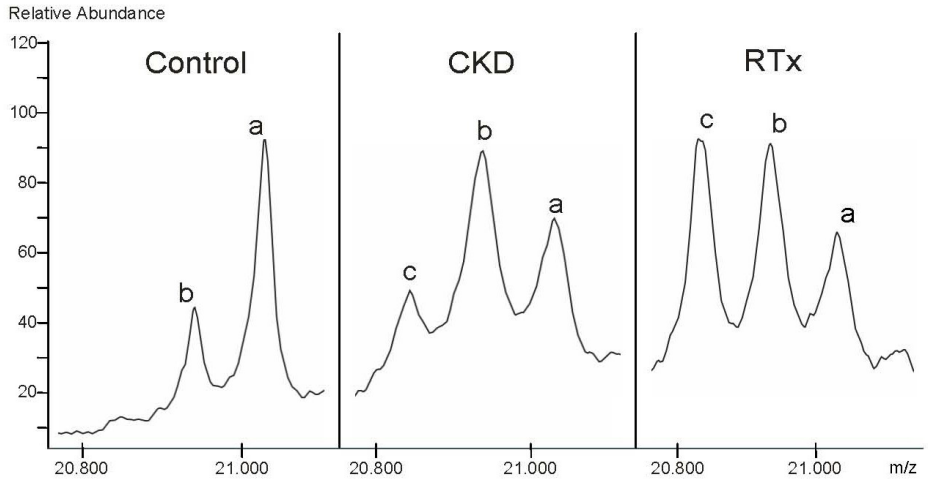

Figure 18 Representative mass spectrometry spectra of RBP4 in a healthy control, a patient with chronic kidney disease (CKD) and a patient after renal transplantation (RTx).
The healthy control shows the non-truncated RBP4 peak (a = 21.065 Da) and the RBP4-L peak (b = 20.950 Da) whereas the CKD and RTx patients also show the RBP4-LL peak (c = 20.837 Da).

Analysis of the TTR mass spectra revealed, that all TTR isoforms (cys-TTR, cysglyc-TTR, glut-TTR) were increased in RTx patients compared to controls whereas glut-

TTR was the only TTR isoform which was increased in CKD patients compared to controls.

In summary, the data indicate that RBP4 and TTR levels are significantly altered in patients with decreased kidney function. In addition, this is the first study evaluating alterations in RBP4 and TTR isoforms in a large cohort of patients with CKD and after RTx showing that RBP4 and TTR isoforms are increased in CKD and RTx patients.

4.1.6 RBP4 and TTR levels but not isoforms are decreased in human subjects with various liver diseases

In the above presented results in CKD and RTx patients it was shown that serum RBP4 and TTR levels and isoforms are affected during kidney dysfunction. Since liver function is also affected in T2DM and obesity and is the site of synthesis of RBP4 and TTR, the aim of this study was to evaluate the impact of liver dysfunction on RBP4 and TTR levels and isoforms. For that reason sera of 50 control subjects and 50 patients with liver diseases were investigated.

Anthropometric and biochemical characteristics of controls and CLD patients are shown in Table 8. There were no differences in gender, age, BMI and eGFR as a parameter for kidney function. Serum C-reactive protein (CRP) and glucose levels were higher in CLD patients compared to controls ($P < 0.01$, both). Total protein was decreased in the CLD group ($P < 0.001$). Standard tests of liver function such as the activity of alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) showed increased levels in the CLD group compared to the values in the control group ($P < 0.001$).

Table 8 Anthropometric and biochemical characteristics of controls and patients with various chronic liver diseases (CLD).

	Controls	**CLD**	**P-Value**
N (% m/ % f)	50 (54 / 46)	50 (60 / 40)	n.d.
Age (years)	54.8 ± 1.0	52.4 ± 1.5	n.d.
BMI (kg/m^2)	24.5 ± 1.3	23.5 ± 0.7	n.d.
Glucose (mmoL/L)	4.73 ± 0.06	5.44 ± 0.27	0.016
CRP (nmoL/L)	1.24 ± 0.88	57.58 ± 9.59	< 0.001
Protein (g/L)	67.0 ± 0.7	50.7 ± 1.1	< 0.001
AST [µkat/L]	0.37 ± 0.02	1.08 ± 0.11	< 0.001
ALT [µkat /L]	0.19 ± 0.02	1.44 ± 0.20	< 0.001
ALP [µkat /L]	1.08 ± 0.06	2.07 ± 0.27	< 0.001
GGT [µkat /L]	0.34 ± 0.03	1.50 ± 0.21	< 0.001
eGFR (mL/ min/ 1.73 m²)	88.1 ± 1.9	93.4 ± 3.7	n.d.
Hb (g/L)	141.2 ± 2.2	145.4 ± 2.9	n.d.
Ht	0.41 ± 0.01	0.42 ± 0.01	n.d.

Data are expressed as mean ± S.E.M. n.d., not different; m, male; f, female; BMI, body mass index; CRP, C-reactive protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma glutamyl transferase; eGFR, estimated glomerular filtration rate; Hb, haemoglobin; Ht, hematocrit, n.d., not different.

With regard to ROH and its transport proteins, it could be shown that serum ROH levels were increased in controls compared to CLD ($P < 0.001$). Moreover, compared to controls, RBP4 levels were lower in CLD ($P<0.001$, Table 9). Higher TTR levels were observed in controls compared to CLD ($P < 0.001$, Table 9). There was no difference in relative amounts of holo-RBP4 and apo-RBP4 of controls and CLD patients. There was a significant correlation of apo-RBP4 and the RBP4-ROH ratio (Spearman Rho $r = 0.565$, $P < 0.01$) indicating an elevation in free RBP4 and thus apo-RBP4 (unbound ROH). In addition, there was no difference in RBP4-L and RBP4-LL in CLD and controls. With regard to the TTR isoforms the cys- and the glut-TTR were significantly increased in the CLD group.

Table 9 Parameters of the RBP4-TTR-complex in serum of controls and patients with various chronic liver diseases (CLD).

Variable	Controls (n = 50)	CLD (n = 50)	P-Value
ROH (µmol/ L)	1.53 ± 0.07	1.08 ± 0.07	< 0.001
RBP4 (µmol/ L)	2.16 ± 0.06	1.18 ± 0.0	< 0.001
TTR (µmol/ L)	6.14 ± 0.19	1.63 ± 0.14	< 0.001
Holo-RBP4 (%)[1]	85.5 ± 1.7	90.3 ± 3.6	n.d.
Apo-RBP4 (%)[1]	9.7 ± 3.7	9.7 ± 3.7	n.d.
RBP4-L (% of non-truncated RBP4)[2]	53.81 ± 6.76	66.29 ± 12.69	n.d.
RBP4-LL (%of non-truncated RBP4) [2]	3.27 ± 1.18	7.79 ± 3.23	n.d.
Cys-TTR (% of native TTR) [2]	236.67 ± 12.49	383.63 ± 25.46	< 0.001
Cysglyc-TTR (% of native TTR) [2]	29.65 ± 2.76	28.65 ± 5.49	n.d.
Glut-TTR (% of native TTR) [2]	44.74 ± 2.67	60.83 ± 4.28	< 0.001

Data are expressed as mean ± S.E.M. n.d., not different; CLD, patients with chronic liver diseases; ROH, retinol; RBP4, retinol-binding protein 4; TTR, Transthyretin; holo-RBP4 / apo-RBP4, RBP4 bound / not bound to ROH; RBP4-L / RBP4-LL, RBP4 truncated at the C-terminal end by one / two leucine molecules; cys-TTR, S-cysteinylated transthyretin; cys-glyc-TTR, S-cysteinglycinylated transthyretin; glut-TTR, S-glutathionylated transthyretin. [1] Holo- and apo-RBP4 were calculated by comparing the intensity of the apo- band to the holo-RBP4 band of each lane (sample) after non-denaturating electrophoresis-immunoblotting and are displayed as percentage of total intensity per lane which has been set 100%. [2] The intensity of the non-truncated RBP4/native TTR peak in the mass spectra was set 100% and the intensities of RBP4 and TTR isoforms are expressed in % of non-truncated RBP4/native TTR.

To further evaluate the impact of specific liver diseases on the RBP4-TTR complex the CLD patients were divided according to the diagnosis of the liver disease and RBP4-TTR parameters analysed. The CLD group was divided into patients suffering from fibrosis (n=34), HCC (n=10) and NAFLD (n=6). With regard to these specific liver diseases, ROH levels were elevated in patients with fibrosis and NAFLD compared to patients with HCC ($P < 0.01$). Highest TTR levels were observed in controls compared to all CLD groups ($P < 0.01$). However, TTR levels were increased in NAFLD patients compared to patients with fibrosis and HCC ($P < 0.05$). The evaluation of RBP4 and RBP4 isoforms according to CLD diagnosis showed that – despite equal eGFR (Figure 19) – RBP4 levels were decreased in NAFLD patients compared to controls ($P<0.05$) but showed a tendency to be higher compared to HCC and fibrosis patients. In addition, RBP4-L was significantly increased in NAFLD patients compared to controls, fibrosis and HCC patients ($P < 0.05$). There was no difference in relative amounts of RBP4-LL levels among the groups (Figure 19).

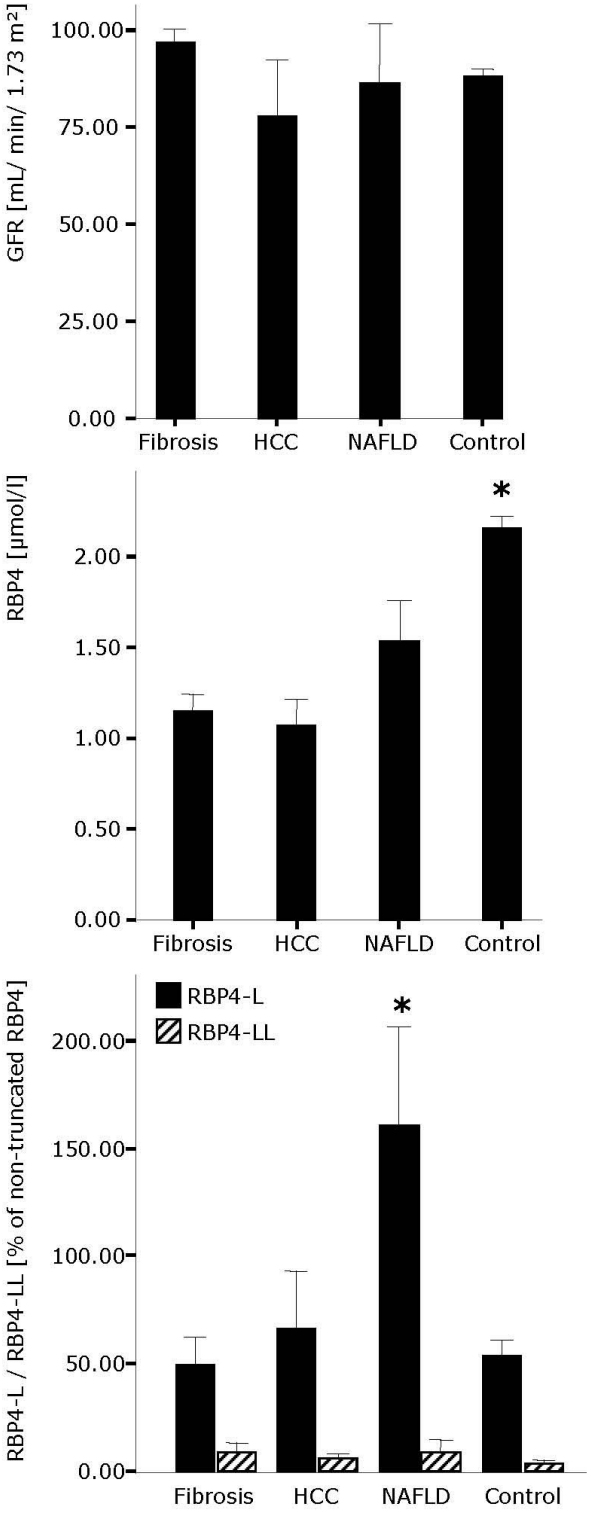

Figure 19 Estimated glomerular filtration rate (eGFR), RBP4 levels and RBP4 isoforms (RBP4-L, RBP4-LL) in patients with fibrosis, hepatocellular cancer (HCC), NAFLD (Non-alcoholic fatty liver disease) and in control subjects.

**, significant different to other groups (P<0.05).*

Taken together, in CLD patients RBP4 and TTR levels are decreased compared to controls. Relative amounts of RBP4 isoforms remain unchanged whereas TTR isoforms seem to be influenced by liver dysfunction. Interestingly, sera of NAFLD patients show different pattern: they have higher RBP4 levels compared to other CLD and show increased amount of RBP4-L.

4.2 Part 2: Investigations on the intracellular retinol-binding protein CRBP-I

CRBP-I belongs to the family of fatty acid-binding proteins which are involved in the intracellular metabolism of ROH. So far, data on the functions of CRBP-I within extra-hepatic cells are limited. In fact, the only known function of CRBP-I is to mediate the transport of ROH to its esterifying enzymes in the hepatocyte (Ghyselinck *et al.* 1999). Since ROH and its metabolites are new players in adipogenesis and insulin sensitivity (Desvergne 2007), the aim of this second part was to investigate extra-hepatic functions of CRBP-I in CRBP-I knock-out (CRBP-I KO) mice.

4.2.1 RE and ROH levels are increased in the adipose tissue but not in the liver of CRBP-I KO mice

To evaluate the effect of the lack of CRBP-I on ROH storage, ROH and RE levels were analysed in serum, liver, pancreas, kidney, lung and two adipose tissues (epididymal and subcutaneous) of CRBP-I KO and WT mice.

Liver tissue levels of ROH and RE were decreased in CRBP-I KO compared to WT mice whereas in epididymal WAT the levels of ROH and RE were increased in CRBP-I KO compared to WT mice ($P<0.001$, both). Moreover, subcutaneous WAT did reveal higher ROH and RE levels compared to WT mice ($P<0.001$ and 0.05, respectively). In the pancreas the amount of RE was decreased in CRBP-I KO compared to WT mice and ROH levels were not different from WT mice (Table 10). In the lung, RE levels were higher in CRBP-I KO compared to WT mice ($P<0.01$).

There were no differences in RE and ROH levels in serum and kidney between CRBP-I KO and WT mice.

Table 10 Retinol (ROH) and retinyl ester (RE) levels in CRBP-I KO and wild-type (WT) mice.

Tissue	WT [µg/g tissue]	CRBP-I KO [µg/g tissue]	P-Value
Serum ROH	38.5 ± 2.4	41.6 ± 2.8	n.d.
Liver ROH	2.8 ± 0.3	0.9 ± 0.0	< 0.001
Liver RE	55.9 ± 8.6	9.5 ± 2.0	< 0.001
WAT ROH	0.6 ± 0.2	1.3 ± 0.0	< 0.01
WAT RE	0.9 ± 0.3	2.8 ± 0.4	< 0.01
subWAT ROH	0.4 ± 0.0	1.5 ± 0.1	< 0.001
subWAT RE	3.5 ± 0.2	5.6 ± 0.8	< 0.05
Pancreas ROH	1.6 ± 0.4	1.1 ± 0.1	n.d.
Pancreas RE	0.9 ± 0.2	0.1 ± 0.0	< 0.05
Kidney ROH	0.3 ± 0.0	0.5 ± 0.1	n.d.
Kidney RE	3.0 ± 0.8	3.4 ± 1.0	n.d.
Lung RE	12.1 ± 3.0	38.8 ± 6.5	< 0.01

WAT, white adipose tissue; subWAT, subcutaneous WAT. Data are displayed as means ± S.E.M.; n.d., not different.

Taken together, CRBP-I seems to be essential to maintain RE stores in the liver and pancreas. In the adipose tissue and the lung other factors apart from CRBP-I may be involved in ROH esterification which enhance in ROH storage.

4.2.2 Dietary hypovitaminosis A does not affect retinylester stores in adipose tissue and pancreas in *CRBP-I knock-out mice*

To further elucidate which compartments of the body are affected by the lack of CRBP-I, mice were fed with a vitamin A deficient (VAD) diet for 8 weeks starting at the age of 9 weeks. The control group was continued to be maintained of a SD for 8 weeks.

At the beginning of the experiment at week 9, when animals were divided into VAD diet and SD group, male CRBP-I KO and WT mice were not different in their weight. After week 3, 4 and 8 the relative weight gain in the control mice on SD was increased in WT compared to CRBP-I KO mice ($P<0.01$). In contrast, in the VAD group CRBP-I KO mice showed a decrease in weight gain after week 3 and 4 ($P<0.05$) but did not show any differences in weight gain afterwards and showed a

tendency towards the same weight gain as WT mice (week 8 on VAD: 112.7% and 103.6% in CRBP-I KO and WT mice, Figure 20).

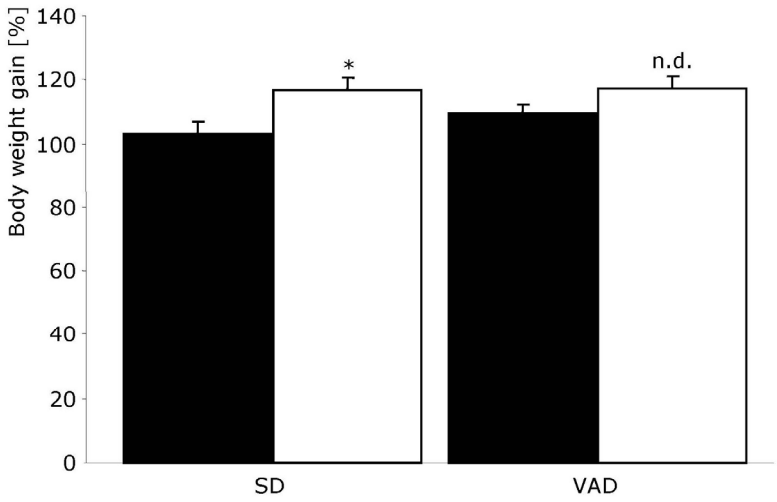

Figure 20 Body weight gain in CRBP-I KO (black column) and wild-type (white column) mice after 8 weeks on standard diet (SD) or vitamin A deficient (VAD) diet.
*Columns are mean and S.E.M.; *, significant different (P<0.05); n.d., not different.*

Liver

With regard to hepatic ROH levels there were no differences detected in ROH levels in livers of CRBP-I KO and WT mice independent of diet (Figure 21). The lack of significant differences is probably due to the small amount of livers used in this analysis (n=2). Hepatic REs stores of CRBP-I KO on SD are lower compared to WT mice ($P < 0.05$) and mice after 8 weeks on VAD diet reveal a dramatic decrease in hepatic RE levels compared to WT mice ($P < 0.01$).

Adipose tissue

In the adipose tissue there was a reduced amount of ROH in WT mice compared to CRBP-I KO mice maintained on the SD as well as on VAD diet. Interestingly, RE levels in adipose tissue of CRBP-I KO mice were higher compared to WT mice independent of diet (SD or VAD, $P<0.05$).

Pancreas

Under dietary hypovitaminosis A there is no difference in ROH levels in the pancreas although CRBP-I KO mice maintained on SD show a reduction in ROH levels in pancreas. There was no change in RE levels in the pancreas of CRBP-I KO mice compared to WT mice on SD or VAD.

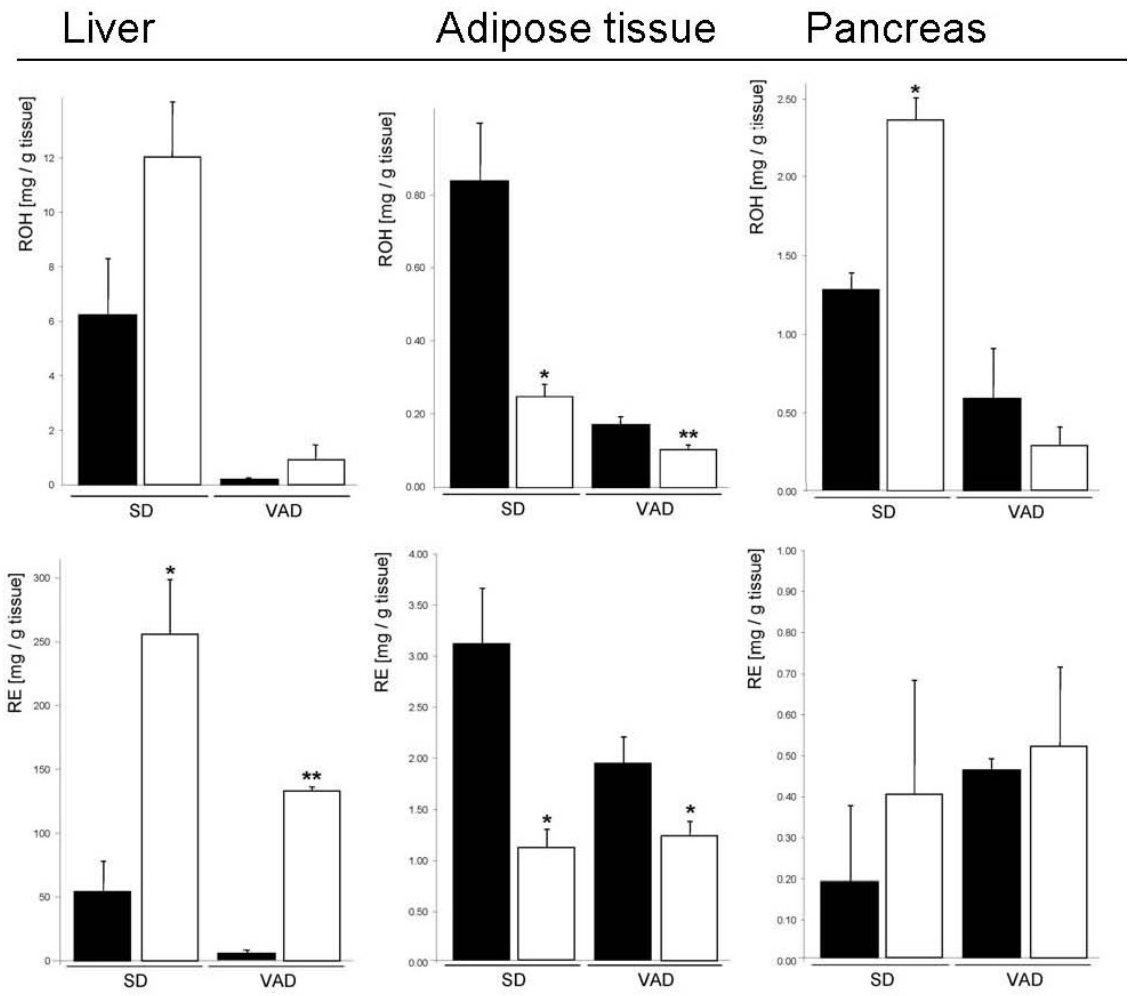

Figure 21 Retinol (ROH) and retinyl ester (RE) levels in the liver, white adipose tissue, pancreas of CRBP KO (black columns) and wild-type (white columns) mice on standard diet (SD) and vitamin A deficient (VAD) diet.
*Columns are mean and S.E.M.; *, significant different to CRBP-I KO mice (P<0.05); **, significant different to CRBP-I KO mice (P<0.01).*

4.2.3 Postprandial uptake of ROH is increased in the pancreas but not the liver and adipose tissue of CRBP-I KO mice

To assess postprandial ROH clearance and ROH uptake into cells in CRBP-I KO mice, the mice were given an oral bolus of peanut oil containing [^{3}H]-ROH. Plasma clearance and the uptake in liver, WAT, pancreas, heart and kidney was monitored 2, 4, 6 and 24 hours after dosing. As depicted in Figure 22, the clearance of [^{3}H]-ROH was not altered in serum and the uptake into liver and adipose tissue did not differ between CRBP-I KO and WT mice independent of time. In contrast, two hours after dosing the amount of [^{3}H]-ROH was significantly higher in pancreas of CRBP-I KO mice compared to WT mice (P<0.01). After four hours there was no difference

although after six and 24 hours the amount of [^{3}H]-ROH was again increased in CRBP-I KO mice (P<0.05, both, Figure 22).

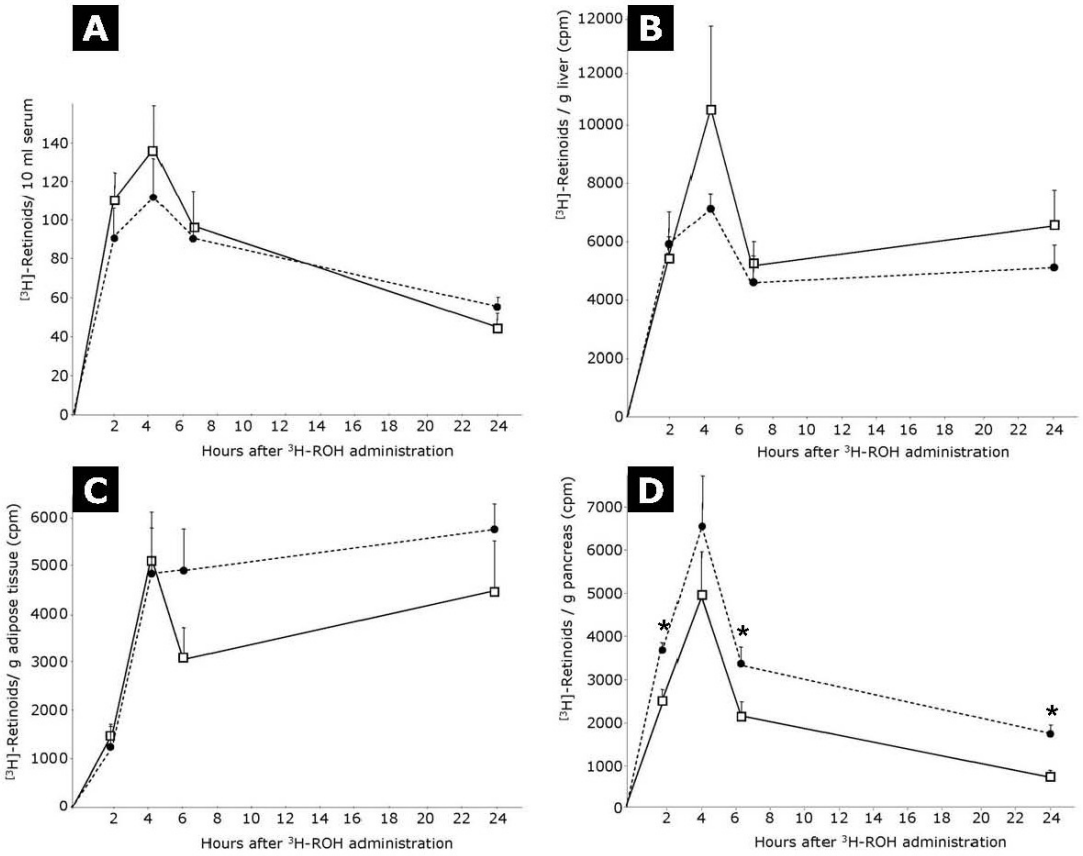

Figure 22 [^{3}H]-Retinoid levels in serum (A), liver (B), adipose tissue (C) and pancreas (D) 2, 4, 6 and 24 hours after an oral bolus of [^{3}H]-ROH in male CRBP-I KO (•) and WT (□) mice.
*[^{3}H]-Retinoid levels were not different in serum, liver and adipose tissue whereas in the pancreas of CRBP-I KO mice [^{3}H]-Retinoid levels after 2, 6 and 24 hours mice were increased compared to WT (indicated by *, P<0.05). n = 4 for each time point and genotype. Values are means ± S.E.M. for each time point.*

Moreover, 24 hours after dosing the amount of [^{3}H]-ROH was increased in the hearts (P<0.01) and in the kidneys of CRBP-I KO mice whereas the increase in the kidneys did not reach significance (P=0.07, data not shown).

4.2.4 Lack of CRBP-I in mice does not alter expression of retinoid metabolizing enzymes

To further evaluate the reasons for the alterations in ROH and RE levels in extra-hepatic tissues, ROH metabolizing enzymes were investigated. Within the cell ROH is bound to CRBPs and is transported to either esterifying enzymes (LRAT, ARAT) or is metabolized to RAL and RA (by ADH or RALDH). Due to the decrease in liver

retinoids and the increase in WAT retinoid levels, it was of interest to analyse whether ROH metabolizing enzymes were altered. Therefore the expression levels of these enzymes were analysed in the liver, the adipose tissue and pancreas of CRBP-I KO and WT mice.

In the liver the relative expression levels of the major ROH esterifying enzyme LRAT as well as ROH oxidizing enzymes (ADH1 and RALDH) were not different. However, RALDH showed a borderline significance towards higher expression levels in CRBP-I KO mice compared to WT mice (P=0.06, Table 11).

Table 11 Expression levels of genes in the liver in wild-type (WT) and CRBP-I KO mice.

Gene	WT	CRBP-I KO
LRAT	1.02 ± 0.22	1.71 ± 0.04
ADH1	1.3 ± 0.3	1.0 ± 0.1
RALDH	0.9 ± 0.1	1.1 ± 0.1

LRAT, lecithin: retinol acyltransferase; ADH1, alcohol dehydrogenase type 1; RALDH, retinaldehyde dehydrogenase type 1. There was no significant difference between WT and CRBP-I KO mice for any gene expression level. Values (relative expression levels) are means ± S.E.M. (n=5 for each genotype).

In the adipose tissue, there were no differences observed in RE metabolizing enzymes (LRAT, ADH1, and RALDH). However, CRBP-III expression was increased in WAT of CRBP-I KO mice compared to WT mice (P<0.05) as well as the expression levels for the RBP4 cell surface receptor STRA6 (P<0.05, Table 12).

Table 12 Expression levels of genes in the epididymal WAT in wild-type (WT) and CRBP-I KO mice.

Gene	WT	CRBP-I KO
LRAT	1.6 ± 0.3	1.7 ± 0.6
ADH1	1.3 ± 0.2	1.5 ± 0.2
RALDH	0.4 ± 0.1	0.5 ± 0.2
STRA6	1.2 ± 0.7	3.0 ± 0.5 *
CRBP-III	0.6 ± 0.1	1.0 ± 0.1 *

*LRAT, lecithin: retinol acyltransferase; ADH1, alcohol dehydrogenase type 1; RALDH, retinaldehyde dehydrogenase type 1. Values (relative expression levels) are means ± S.E.M. (n=5 for each genotype). *, significant different (P<0.05).*

Moreover, in the pancreas there were no differences in expression levels of RALDH and ADH1.

4.2.5 Enzymes involved in triglyceride synthesis may also catalyse retinylester synthesis in adipose tissue

To give an explanation for the higher ROH and RE levels in adipose tissue the expression levels of metabolizing enzymes were analysed. Since there were no differences in ROH metabolizing enzymes (LRAT, ADH1 and RALDH) in WAT, one might speculate that other enzymes must be capable of carrying out ROH esterification. In fact, it is known that ROH esterification is catalysed by the enzyme LRAT. The second possibility for ROH esterification is the ARAT reaction. To that date it remains unknown which enzymes catalyse the ARAT reaction. However, there is evidence that enzymes of the monoacyglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase (DGAT) family might be capable to esterify ROH to RE. MGAT and DGAT belong to a family of enzymes which mediate triglyceride synthesis by mediating the acylation of monoacylglycerol and diacylglycerol (Shi and Cheng 2008). The isoforms MGAT1 and MGAT2 of the MGAT family are expressed in several tissues including adipose tissue (Orland *et al.* 2005; Shi and Cheng 2008). The isoform DGAT1 of the DGAT family is mainly expressed in the small intestine (Shi and Cheng 2008) but also adipose tissue (O'Byrne *et al.* 2005). Due to the lack in differences mRNA levels of ROH esterifying enzymes in the WAT, the enzymes MGAT1, MGAT2 and DGAT1 were tested for their ability to esterify ROH to RE. Thus, HEK293 cells, which have been used previously as a cell culture model to study ROH metabolism (O'Byrne *et al.* 2005), were transfected with the vector pcDNA3 as a control and the vector encoding genes for human MGAT1, MGAT2 and DGAT1. Successful transfection was verified by real-time PCR (data not shown). ROH was then added to the medium and after incubation ROH and RE levels were analysed in the cells.

Figure 23 shows that all cell samples were able to take up ROH into the cell. MGAT1 was able to strongly esterify ROH which was taken up into the cell to RE as well as DGAT1 ($P<0.05$, both). Moreover, ROH levels were significantly decreased in DGAT1 transfected cells compared to control cells.

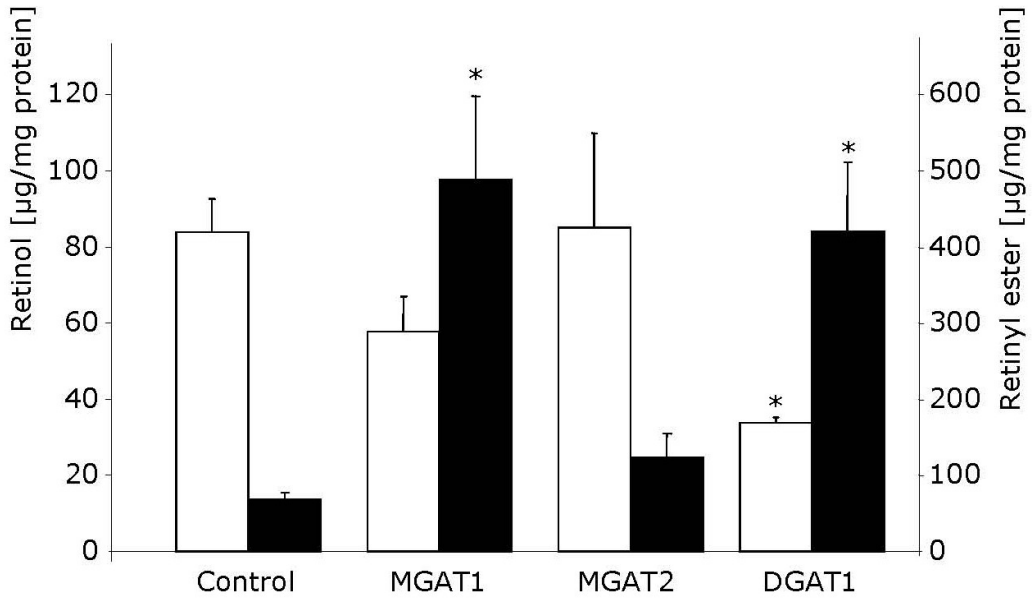

Figure 23 Retinol (white column) and retinylester (black column) levels in HEK-293 cells after transfection with MGAT1, MGAT2 and DGAT1 vectors.
*MGAT1 and MGAT2, monoacylglycerol acyltransferase type 1 and 2; DGAT1, diacylglycerol acyltransferase type 1. Data are presented as means and S.E.M.; *, indicates significant differences compared to control cells (P<0.05).*

MGAT1, MGAT2 and DGAT1 expression was also detected in adipose tissue of CRBP-I KO and WT mice. To sum up, the enzymes MGAT1 and DGAT1 which are known to be involved in synthesis of triglycerides are able to synthesize RE from ROH and are expressed in adipose tissue of CRBP-I KO and WT mice.

4.2.6 Lack of CRBP-I decreases body weight in mice

To elucidate extra-hepatic functions of CRBP-I in mice, CRBP-I KO mice were generated and maintained on a SD. The mice were weighed once a week for 19 weeks starting at week 5 after the date of birth. At week 6 the relative weight gain in CRBP-I KO mice was higher compared to WT mice (P=0.02). Beginning with week 21 the weight gain in CRBP-I KO mice was reduced compared to WT mice and remained decreased until the end of the experiment at the age of 24 weeks (P=0.05, Figure 24).

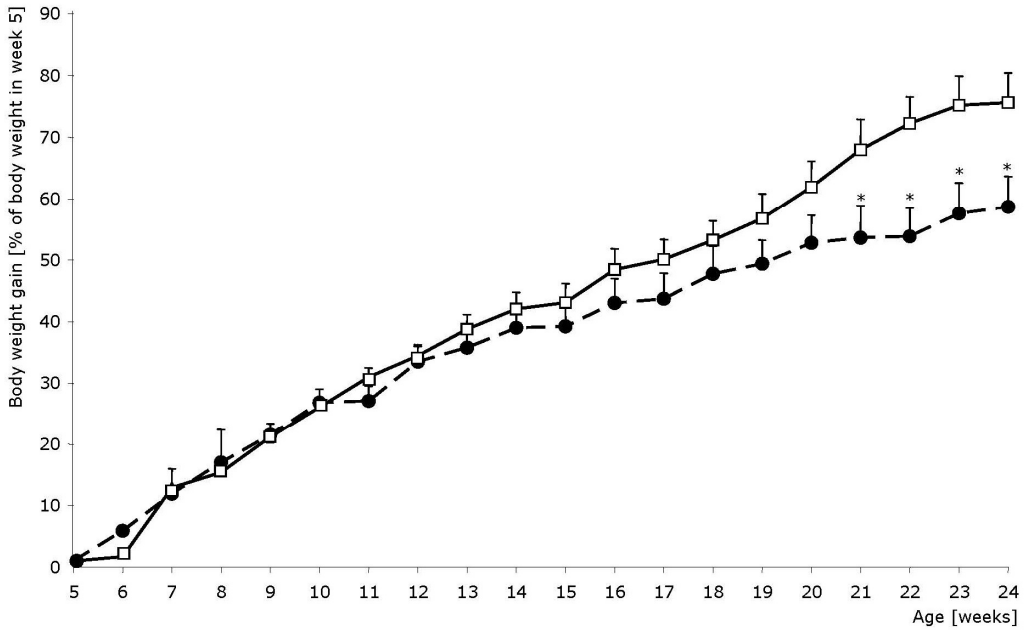

Figure 24 Increase in body weight in per cent of initial body weight (BW) in wild-type (WT, □, n=6) and CRBP-I KO (●, n=7) mice fed a SD.
*BW was decreased in CRBP-I KO mice compared to WT mice at the age of 21 to 24 weeks (indicated by *, P<0.05). Data are presented as means and S.E.M.*

At the age of 21 and 37 weeks, the weight of the epididymal fat pad was evaluated in 12 CRBP-I KO and 16 WT mice (expressed as percentage of body weight). Although body weight was higher in WT compared to CRBP-I KO mice at the age of 21 and 37 weeks (Figure 25 A), the relative amount of epididymal body fat was not different in CRBP-I KO compared to WT mice (Figure 25 B).

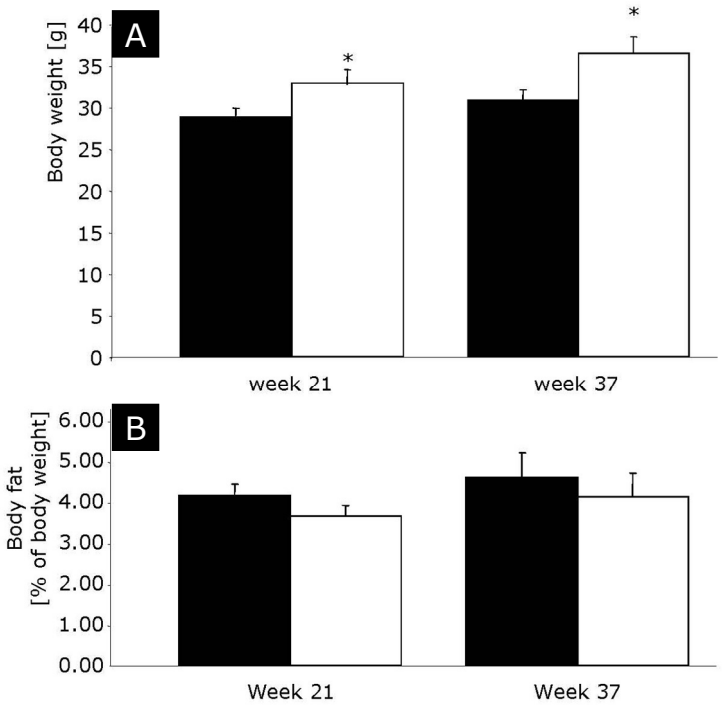

Figure 25 Body weight (A) and relative amount of epididymal body fat (B) in CRBP-I KO (black bars, n=6) and WT (white bars, n=8) mice at the age of 21 and 37 weeks.
*Data are presented as mean and S.E.M. *, significant different (P<0.05).*

4.2.7 Lack of CRBP-I alters serum triglyceride levels

ROH esterification is strongly connected with fatty acid esterification, since both, ROH and 2-monoacylglycerols, are transported by chylomicrons to the liver after intestinal absorption (Orland *et al.* 2005). To that date investigations on the functions of CRBP-I were restricted to hepatic metabolism. Therefore, in this study triglyceride as well as levels of free fatty acids were examined. At the age of 21 weeks the levels of free fatty acids were decreased in CRBP-I KO mice compared to WT mice (0.63 ± 0.09 and 0.87 ± 0.06 µmol/L, respectively, $P<0.05$). In contrast, at the age of 37 weeks there was no difference in free fatty acids (0.62 ± 0.07 and 0.60 ± 0.07 µmol/L). At the age of 21 weeks, triglyceride levels were not different between the genotypes (0.44 ± 0.05 and 0.44 ± 0.06 mg/dL) whereas at the age of 37 weeks triglyceride levels in CRBP-I KO were lower compared to WT mice (0.46 ± 0.05 and 0.91 ± 0.05 mg/dL, respectively, $P<0.01$).

4.2.8 Despite comparable fasting glucose levels CRBP-I KO mice reach initial glucose levels faster after insulin administration

CRBP-I has been reported to be expressed in high amounts in pancreas compared to other tissues (Kato *et al.* 1985). Therefore, serum glucose was evaluated after fasting and an insulin tolerance test was performed to evaluate alterations in glucose metabolism. After 6 hours of fasting serum glucose levels were not different between CRBP-I KO and WT mice (172 ± 9 and 177 ± 9 mg/dl, respectively, Figure 26).

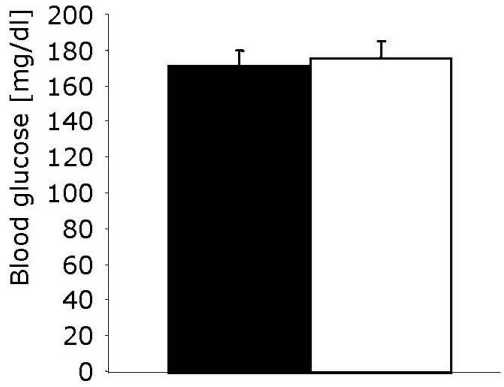

Figure 26 Blood glucose levels after 6 hours of fasting in CRBP-I KO (black column, n=6) and WT mice (white column, n=8).
Data are presented as means and S.E.M.

To test the tolerance for insulin and the capacity of peripheral tissues to uptake glucose, the mice were administered an intraperitoneal dose of insulin. After 15 minutes blood glucose values were fallen by more than 30 per cent in CRBP-I KO and WT mice. The lowest glucose values were observed 45 minutes after insulin administration. 90 minutes after insulin administration the increase in blood glucose levels was more pronounced in CRBP-I KO compared to WT mice (Figure 27).

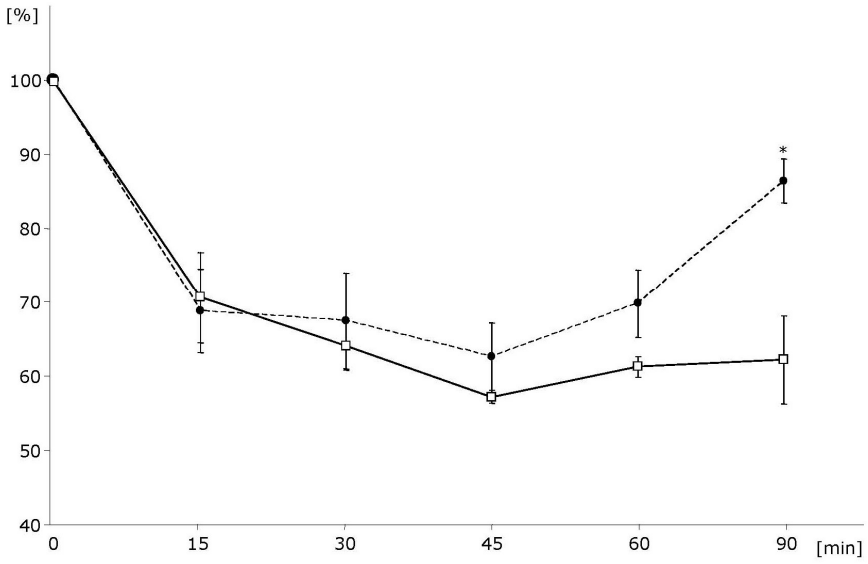

Figure 27 Decrease in blood glucose levels in per cent of initial value after intraperitoneal insulin injection in CRBP-I KO (•, n=6) and WT mice (□, n=8).
*Values are means ± S.E.M. *, significant different (P < 0.05).*

5 Discussion

RBPs have been reported to play a role in insulin resistant states and retinoids have been postulated to be new players in adipogenesis. To that date the data in this new field of research remain limited. Therefore the first part of this work investigated the extracellular ROH transport proteins, RBP4 and TTR. The second part focuses on the extra-hepatic functions of the intracellular ROH binding protein CRBP-I.

5.1 Extracellular retinol binding proteins in humans and mice

5.1.1 Tissue specific alterations in the adipose tissue, the kidney and the liver during obesity

For a long time the main function of adipose tissue has been regarded to be the storage of triglycerides. In fact, adipocytes are capable of storing large amounts of triglycerides reflecting excess energy and are capable of releasing lipids in times of need. However, apart from weight gain excess of dietary fat also leads to an expansion of adipose tissue. During obesity adipocytes enlarge and macrophages are infiltrating between the cells. In times of excess caloric intake the increased input of triglycerides in the adipocyte can be balanced by an increased triglyceride beta-oxidation in the muscle (Guilherme *et al.* 2008). However, during long-term overload of adipocytes the recruitment of macrophages into adipose tissue causes an inflammatory state and the release of free fatty acids into the circulation (Wang *et al.* 2008). Indeed, the adipose tissue of WT mice on HFD and of leptin-deficient *ob/ob* mice did show an enlargement of adipocyte size (hypertrophy). Moreover, the observed increase of infiltrated macrophages in the WAT of these animals indicates an inflammatory state and is associated with decreased insulin sensitivity (Jurgens *et al.* 2007). In the majority of T2DM patients the disease is the result of an interaction between genetic predisposition and lifestyle (Ritz *et al.* 1999). Therefore the diet-induced obesity in WT mice may reflect the "lifestyle" part in the development of the disease.

Interestingly, adipose tissue also plays a role in ROH metabolism since up to 20% of whole body retinoids may be stored there and significant amounts of RBP4 mRNA can be found. Tsutsumi and Okuno (1992) postulated therefore that adipose tissue may contribute the whole body RBP4 production and pools. During the last decade

research has focussed on proteins that are secreted by adipose tissue: the adipokines. RBP4, the transport protein for ROH in the serum, has been reported to be a new adipokine beside others such as leptin and adiponectin. However, the regulation of RBP4 from adipocytes is still not known as well as whether RBP4 serum levels are regulated at the level of transcription, translation or secretion (Tamori *et al.* 2006). Moreover, it is not known if the adipocyte derived RBP4 is bound to ROH (holo-RBP4) or if it circulates freely as apo-RBP4. Therefore one might speculate that if there is not enough ROH within the adipocyte, RBP4 may be secreted without ROH as apo-RBP4 (Mills *et al.* 2008).

It has been shown that RBP4 mRNA levels are higher in visceral than subcutaneous adipose tissue and that serum RBP4 shows a strong correlation with the RBP4 expression in the white adipose tissue. The increased RBP4 expression in visceral fat might contribute to the development of insulin resistance due to the release of RBP4 in the portal circulation (Kloting *et al.* 2007). However, during insulin resistance and increased macrophage infiltration in stromal vascular cells no significant increase of RBP4 mRNA was observed (Kloting *et al.* 2007). This indicates in turn that macrophages are unlikely to contribute to the increase in RBP4 levels.

Although it could be shown in this work that RBP4 and TTR are secreted into medium by primary adipocytes*, s*taining of RBP4 and TTR in adipose tissue was not observed in this experiment. The reason for the lack of staining might be due to the morphology of the adipocytes itself. It contains one large fat vacuole which displaces the cytoplasm to the side of the cell (Schiebler 1996). Therefore the relative part which could possibly be stained for RBP4 or TTR in the adipocyte might be too small and explain the lack of staining. In addition, during the preparation of the sections the cytoplasm of the adipocytes is often destroyed and leads to a damage in the cell structure which may initiate the catabolism of RBP4 (Schiebler 1996).

Moreover, it is known that in the liver RBP4 is not stored but released after forming a complex with ROH (Kato *et al.* 1984). This is supported by the fact that the mice were maintained on diets containing 15 IU ROH/g diet indicating that the uptake of ROH might be sufficient and lead to a release of RBP4 and ROH into the circulation. Moreover, the analysis of RBP4 and TTR expression in adipose tissue of WT on chow

and HFD as well as in *ob/ob* mice in this work did not reveal any differences. This is probably due to the small sample size of n = 2 of each group.

Summing up, the data indicate that in the adipose tissue RBP4 release is probably similar regulated as in the liver: the protein is expressed independently of ROH availability but released very rapidly in times of adequate ROH amounts.

With regard to the kidney – the site of RBP4 reabsorption – strong RBP4 staining was observed. The kidneys play in important role in the catabolism of RBP4 since the protein is catabolized in the proximal tubular cells because it is filtered by the renal glomeruli (Kato *et al.* 1984). In the case of kidney dysfunction the loss of functional kidney tissue leads to an increased half-life of RBP4 in the circulation. The RBP4 staining of proximal tubular cells in the presented sections represent RBP4 that has undergone glomerular filtration and tubular reabsorption (Kato *et al.* 1984). It has been suggested that apo-RBP4 undergoes catabolism in the kidneys and is not reutilized. However, in the kidneys there is also endogenous RBP4 expression (5-10% of liver expression). This is due to the fact, that ROH is reabsorbed via a megalin-mediated process in the kidneys, the recycling and reutilization of ROH is dependent on newly synthesized RBP4 in the kidneys which explains the expression of RBP4 in the kidneys (Soprano *et al.* 1986). Therefore the observed RBP4 staining may be the result of reabsorbed RBP4 and also of newly synthesized RBP4. The microcirculation of the kidneys is often affected in obesity and T2DM. The destruction of functional kidney tissue in older mice with obesity has been shown representatively in this study and has been reported in the literature (Ritz *et al.* 1999; Morcos *et al.* 2002). The very mild staining for TTR in the kidneys is consistent with the fact that TTR is only weakly catabolized in the tubular cells due to the glomerular barrier and staining is mostly the result of intracellular TTR expression (Waldmann *et al.* 1972; Kato *et al.* 1984).

The liver sections of WT mice on HFD and *ob/ob* mice did show an accumulation of lipid droplet in the cytoplasm of cells accompanied with a destruction of liver architecture. It has been reported previously that a HFD leads to an increase in hepatic triglyceride levels (Zizola *et al.* 2008). Due to an increased up-take of non-esterified free fatty acids into the liver during excess of energy in obesity, the storage of triglycerides in hepatocytes contributes to the development of fatty liver and insulin resistance (Blaner *et al.* 2008). Moreover, the liver plays a central role in the uptake, storage and mobilization of ROH in the body (Kato *et al.* 1984) since

approximately 70% of total body retinoids are stored in the liver. In the case of liver injury ROH storage cells, the stellate cells, become activated, convert into fibrogenic myofibroblasts and lose their RE containing lipid droplets (Blaner *et al.* 2008). Hepatic secretion of RBP4 is regulated by the availability of ROH, i.e. in ROH deficiency RBP4 synthesis continues but RBP4 is not secreted (Selvaraj *et al.* 2008). This leads to the accumulation of RBP4 in the endoplasmatic reticulum of the hepatocyte, since the secretion but not the synthesis is dependent on ROH (Ronne *et al.* 1983; Noy 2000). The mice in this work, however, were maintained on a ROH containing diet indicating normal secretion of RBP4 from the liver. Although dietary ROH intake was normal, the liver injury of WT mice on HFD and the *ob/ob* mice might disturb the hepatic ROH and RBP4 homeostasis and explain the mild staining for RBP4 in these mice.

TTR, like RBP4, is synthesised in the liver and is secreted with RBP4 and ROH into the circulation but does not accumulate in the hepatocyte during excess or deficiency of ROH (Kato *et al.* 1984). This is consistent with the results in this work showing mild staining for TTR. Within that context, it is an interesting fact that RBP4 and TTR are localised in the parenchymal cells whereas CRBP-I is localised mainly in the hepatic fat-storing cells[6] (Kato *et al.* 1984). Therefore staining for RBP4 and TTR seems to remain unchanged during obesity indicating that synthesis of these proteins is not altered.

5.1.2 Expression and secretion of RBP4 and TTR in adipocytes

Adipose tissue is, next to the liver, important in retinoid uptake, accumulation and metabolism (Tsutsumi *et al.* 1992). To that date, it is known that RBP4 is expressed in WAT but the regulation remains unclear. First, previous results on RBP4 levels in WAT of WT and *ob/ob* mice were confirmed showing higher RBP4 levels in *ob/ob* mice (Yang *et al.* 2005). *ob/ob* mice have been extensively studied with regard to obesity. However, *db/db* mice have not been studied yet in relation to RBP4. Secondly, the RBP4 expression in *db/db* mice was found to be not as strong as in *ob/ob* mice. *db/db* mice are a model for T2DM and dyslipidemia (Srinivasan and Ramarao 2007). The goal of the experiments in 3T3-L1 and primary adipocytes was to investigate RBP4 and TTR expression and secretion in these cell culture models

[6] Fat-storing cells are also called stellate cells, lipocytes, vitamin A storing cells and Ito cells. Kato, M., K. Kato and D. S. Goodman (1984). "Immunocytochemical studies on the localization of plasma and of cellular retinol-binding proteins and of transthyretin (prealbumin) in rat liver and kidney." J Cell Biol **98**(5): 1696-704.

and to explore the mechanisms of ROH efflux from adipose tissue after treatment with ROH, RA and insulin. Treatment with RA is of interest since RA occurs naturally in adipose tissue and can influence adipocyte differentiation in vitro (Desvergne 2007). Insulin treatment may influence RBP4 expression since increased RBP4 serum levels were observed in T2DM patients (Graham *et al.* 2006). It was shown in this work that 3T3-L1 adipocytes as well as primary mouse adipocytes express and also secrete RBP4 and TTR. This indicates that adipocytes are not only able to accumulate ROH from the circulation but are probably also able to secrete ROH back into the circulation. However, the experiments also demonstrate that 3T3-L1 cells are not a useful model to study RBP4 and TTR since mRNA and protein levels were very low. The low concentration of RBP4 mRNA in 3T3-L1 cells has been reported previously by others (Mercader *et al.* 2008). The studies in primary adipocytes showed that RBP4 and TTR are expressed. However, the lack of differences in RBP4 and TTR expression following different treatments may be due to the short period of incubation (6 hours) with ROH, RA and insulin. In fact, mRNA levels of CRBP-I have been shown to increase significantly after incubation (with RA) for 24 hours but not RBP4 levels (Okuno *et al.* 1995). In contrast, a decrease of RBP4 mRNA was observed in another study after incubation with RA for 48 hours (Mercader *et al.* 2008).

With regard to secretion, the data of this work show that RBP4 secretion was not altered after treatment with ROH, RA and insulin. This is in agreement with an experiment carried out by Okuno and Caraveo (1995) who treated adipocytes with 1µM RA and did not find changes in RBP4 mRNA as well as protein levels. Interestingly, CRBP-I expression has been reported to be increased after physiological concentrations of RA indicating that RBP4 and CRBP-I expression are not regulated in a coordinate manner in adipocytes (Okuno *et al.* 1995). Studies by Soprano and Smith (1982) on hepatic RBP4 secretion showed that dietary ROH and RA did not influence RBP4 mRNA in the liver. This seems also to be the case in the experiment of this work. However, although there is evidence that adipocytes secrete ROH it remains unclear, whether ROH is necessary for RBP4 secretion and whether RBP4 is mainly secreted as holo- or apo-RBP4 from adipose tissue. Moreover, the ROH-RBP4 secretion from the liver is fulfilled by the binding to TTR (Gottesman *et al.* 2001). It is not known if the ROH is secreted bound to TTR and RBP4 from adipose tissue. However, there is evidence that the T2DM related effect of RBP4 is caused by apo-RBP4 and not by the liver secreted holo-RBP4 (Aeberli *et al.* 2007). In that case it is unclear how apo-RBP4 which has a very short half-life in

the circulation might act. In addition, it is of interest whether RBP4-ROH is bound to TTR, since RBP4, which is not bound to TTR, is rapidly filtered and degraded in the kidneys. To that date, it remains unclear if ROH is secreted in complex with RBP4 and TTR. However, the experiments here show that TTR is secreted into the medium by primary adipocytes and in higher amounts after treatment with ROH. It has been reported that ROH efflux from adipocytes is triggered by intracellular cAMP levels which regulate fatty acid levels and efflux from adipocytes (Wei *et al.* 1997). However, the relation to TTR secretion remains unknown.

5.1.3 Effect of diabetes on RBP4 and creatinine levels in mice

RBP4 levels have been studied extensively in several mouse models in previously published experiments. However, none of these studies did investigate serum creatinine levels next to RBP4 levels. In the here reported experiment the aim was to evaluate RBP4 levels and creatinine levels in diabetic *db/db* mice. The *db/db* mouse strain is characterised by a mutation in the *db* gene encoding the leptin receptor and is therefore suitable to study obesity and diabetes (Srinivasan and Ramarao 2007). The exposure to a HFD accelerates the onset of obesity and diabetes in the *db/db* mice (Jurgens *et al.* 2007). In fact, the *db/db* mice on SD and HFD had high glucose levels compared to WT mice and HFD did fasten the onset of diabetes. The results of this experiment showed that RBP4 serum levels as well as serum creatinine levels are increased in diabetic *db/db* mice. Apart from the elevation in RBP4 serum levels, body weight and glucose levels were increased in *db/db* mice compared to WT mice. This is in agreement with other studies which have been investigating the relation of elevated RBP4 levels in obesity and diabetes in adipose-*GLUT KO*, *GLUT4*-Tg, WT mice on HFD (Yang *et al.* 2005) and *ob/ob* mice (Mody *et al.* 2008). So far, RBP4 levels have not been investigated in *db/db* mice. Moreover, the parallel elevation of serum creatinine as a marker for kidney function as well as the correlation of RBP4 and creatinine shows that the dysfunction of the kidneys in these mice might contribute to the elevation of RBP4. It is well known, that RBP4 serum levels are elevated during kidney dysfunction. In particular, the decreased ability of the kidneys to filter low-molecular weight proteins leads to an abnormal retention of small serum proteins and to an increase in serum levels of these proteins (Vahlquist *et al.* 1973; Bernard *et al.* 1988; Ziegelmeier *et al.* 2007; Frey *et al.* 2008). The increase in serum creatinine levels underlines that the function of the kidneys in these mice was impaired and due to the close relation between insulin resistance during kidney disease, both, the

increased insulin levels as well as RBP4 levels might be the result of kidney dysfunction. A study carried out by this institute in human CKD patients with and without T2DM underlines this fact: It was shown that rather kidney function determines RBP4 levels than obesity and T2DM (Henze *et al.* 2008). Moreover, in chapter 4.1.2 it was shown that the relative RBP4 levels in adipose tissue of *db/db* mice were not significantly increased compare to WT mice. This indicates that the increase of serum RBP4 is probably not contributing to an increased secretion from the adipose tissue.

In this experiment RBP4 was also associated with body weight which is consistent with other studies (Promintzer *et al.* 2007). Moreover, there was a correlation of RBP4 and insulin levels which has been reported previously in WT mice on HFD (Yang *et al.* 2005).

5.1.4 Effect of obesity and T2DM on RBP4 and TTR in humans

Several human and animal studies have investigated the influence of circulating RBP4 levels in the pathogenesis of insulin resistance associated with T2DM and obesity (Graham *et al.* 2006; von Eynatten *et al.* 2007; Lewis *et al.* 2008). To that date research has not focussed on the metabolism of TTR, the physiological binding protein of RBP4 in the blood. Alterations of TTR-RBP4 interaction could play an important role in this context by stabilizing RBP4 at higher steady-state concentrations and thus impair the renal RBP4 clearance (Mody *et al.* 2008). Therefore, it was of interest to determine RBP4 as well as TTR levels in overweight and overweight T2DM subjects. Since data on isoforms of RBP4 and TTR is limited to results in patients with kidney dysfunction, the aim of this study was also to investigate RBP4 and TTR isoforms in these patients. Moreover, the amount of holo- and apo-RBP4 as possibly influencing factors of RBP4-TTR interaction as well as kidney function in plasma of human subjects was evaluated.

Effect on RBP4 LEVELS

The findings show that levels of both, TTR and RBP4, were not altered in overweight subjects and overweight subjects with T2DM. With regard to RBP4, the results of this work confirm previous investigations showing that circulating RBP4 seems not to be not related to insulin resistance in overweight human subjects (von Eynatten *et al.* 2007; Lewis *et al.* 2008). Graham and Wason (2007) suggested that Western Blot (using recombinant His-tagged RBP4) is the gold standard for the

RBP4 analysis because other methods such as ELISA would underestimate RBP4 levels. The reason for that, they argued, would be the use of urinary RBP4 as a standard which is modified by carboxyl terminus-proteolysis. In this work ELISA was used to analyse RBP4 levels in serum. However, with regard to the above mentioned problem, it is important to mention that this institute uses a RBP4 standard which was isolated from human serum and does contain non-truncated, full-length RBP4[7]. Thus the standard curve is based on the values of non-truncated RBP4 and would not underestimate RBP4 levels. Moreover, some investigators did compare the RBP4 values obtained from ELISA and did not find a difference to the values obtained from Western Blots (Bahr *et al.* 2008).

The lack of differences in RBP4 levels might also be contributed to the age of the study group. It has been reported, that there was a correlation of RBP4 and insulin sensitivity, percent body fat and dyslipidemia in subjects aged 20 to 50 but not in elderly subjects aged 60 to 80 (Gavi *et al.* 2008) – the age which corresponds more to study group in this work (age: 52 - 60 years). In addition, levels of both, RBP4 and eGFR, were similar in the studied subgroups, confirming previous findings, that in the presence of normal renal function, RBP4 is not affected by insulin resistance (Ziegelmeier *et al.* 2007; Henze *et al.* 2008).

Moreover, von Eynatten and Lepper (2007) suggested a role of RBP4 in lipid metabolism instead of a role in the metabolic syndrome. The authors found strong correlations between RBP4 and cholesterol, LDL-cholesterol, VLDL and triacylglycerol whereas no correlation between RBP4 and HOMA-IR was found. This is coherent with the correlations found in this cohort: RBP4 correlated strong with triacylglycerol ($r=0.283$) and WHR (0.256, $P<0.05$, both) but not with HOMA-IR ($r=0.106$). The correlation between serum RBP4 and WHR has been reported previously (Kloting *et al.* 2007). In a different study RBP4 and related changes in its levels predicted alterations in triacylglycerol but not in HDL and LDL levels (Goodman *et al.* 2008). Therefore one might speculate that RBP4 is more related to lipid metabolism than to glucose or insulin metabolism.

Effect on RBP4 ISOFORMS

MALDI-TOF mass spectrometry after immunoprecipitation of RBP4 allows the identification and measurement of posttranslational modification of the RBP4

[7] The full-length form of RBP4 in the standard used for he ELISA was verified by MALDI-TOF mass spectrometry.

molecule: the spectra display the peak for non-truncated RBP4, the peak for RBP4 truncated at the last C-terminal leucine (Leu-183) and the peak for RBP4 truncated at the last two leucine (Leu-182) molecules (RBP4-L and RBP4-LL, respectively). RBP4-L and RBP4-LL were not different in controls and overweight T2DM patients. This is consistent with the hypothesis that RBP4-L and RBP4-LL occur specifically in the serum of patients with restricted kidney function due to a decreased clearance by the kidney (Jaconi *et al.* 1996). In fact, kidney function in overweight and overweight T2DM subjects was not different from kidney function of controls (displayed as eGFR in Table 4 on page 45).

Effect on TTR LEVELS

In the discussion of RBP4 as a potential adipokine in the pathogenesis of insulin resistance, factors that interfere with RBP4 should be considered. With regard to TTR, it has traditionally been seen not only as carrier for both, ROH and thyroxin, but also as a biomarker for nutritional status. This is due to its hepatic synthesis which is mainly influenced by the adequacy of dietary protein and energy intake (Ingenbleek and Young 1994). In the here presented study, plasma TTR levels of overweight and overweight T2DM subjects correspond with those of other investigations which assessed TTR levels as nutritional biomarker (Cano *et al.* 1988; Ferard *et al.* 2002; Gericke *et al.* 2005) and in T2DM subjects (Basualdo *et al.* 1997; Raila *et al.* 2007) showing that TTR was not affected in T2DM. Other studies have shown that TTR levels are elevated in subjects with impaired glucose tolerance or T2DM and that TTR is positively correlated with triglycerides (Yoshida *et al.* 2006; Kloting *et al.* 2007). Therefore, TTR was suggested as a marker of overnutrition (Yoshida *et al.* 2006). However, the results here do not support this relation due to the absence of differences in TTR concentrations among overweight and overweight T2DM subjects. Moreover, the molar ratio of plasma RBP4 to TTR showed that the stoichiometry of these proteins was not altered in overweight T2DM subjects compared to overweight and lean controls. In healthy states, 1 mol of RBP4 binds 1 mol of TTR tetramer, thus the total molar concentration of plasma TTR exceeds that of RBP4 by 2.5–3.5 times (Zago *et al.* 2002). This is consistent with the results presented in this work and indicates that RBP4/TTR stoichiometry is not affected by T2DM.

TTR is important in maintaining circulatory levels of holo-RBP4 because it forms a large transport complex which reduces the glomerular filtration of the relatively small holo-RBP4 molecule and prevents therefore its excessive loss in the urine

(Goodman 1984). Moreover, TTR has a higher affinity to holo-RBP4 compared to apo-RBP4 due to the hydroxyl end group in the ROH molecule, which participates in the H-bond interactions with the TTR molecule (Raz *et al.* 1970). Therefore one can speculate that an elevation of holo-RBP4 may be stabilised by TTR binding and that this binding prevents RBP4 clearance. Indeed, the majority of the studies investigating the role of RBP4 in obesity did not investigate ROH levels, although ROH is the most important companion of RBP4. However, apo-RBP4 has been reported to be elevated in obese subjects (Mills *et al.* 2008). Moreover, the correlation between RBP4 and ROH was stronger in control subjects compared to obese subjects indicating an abnormal RBP4 regulation or metabolism (Mills *et al.* 2008). This might represent a potentially involved mechanism leading to an elevation of circulating RBP4. It was shown in this work that neither the total amount of RBP4 nor the relative amount of holo-RBP4 was altered in both overweight subjects and overweight subjects with T2DM. The amount of holo- to apo-RBP4 is ~85% to ~15% in these patients, which is in agreement with the amounts in healthy subjects (Sapin *et al.* 2000). Nevertheless, the ratio changes under certain conditions such as CKD due to a decreased apo-RBP4 catabolism and thus an increase of apo-RBP4 in the serum (Jaconi *et al.* 1996).

Effect on TTR isoforms

In the present study, posttranslational modifications of the TTR molecule were assessed which allow the elucidation of the modified structures (Gericke *et al.* 2005). It has been reported in a recently published study that structural modifications of TTR might also be involved in RBP4 retention (Mody *et al.* 2008). The detection and characterization of such TTR modifications might be of importance not only for clinical diagnostics but also for the pathogenesis of the disease (Sass *et al.* 2003; Zhang and Kelly 2003; Gericke *et al.* 2005). However, to that date modifications in the TTR molecule have only been reported in relation to the incidence of amyloidosis (Kingsbury *et al.* 2008). For TTR immunoprecipitation RBP4 anti-body was used and thus TTR was co-immunoprecipitated with RBP4. The TTR spectra therefore represent TTR which is bound to RBP4. In general, TTR was dominant in four variants, corresponding with previous studies by others and by this research group (Kiernan *et al.* 2003; Schweigert *et al.* 2004; Gericke *et al.* 2005). All molecular variants including the unmodified native, cys-, cysglyc- and glut-TTR were detected in serum of controls and also in serum of overweight T2DM subjects. Although there was no difference in peak-height ratio of TTR isoforms, the results demonstrate that all co-precipitated TTR isoforms were involved in binding

to RBP4. This is due to the fact that TTR was immunoprecipitated by RBP4 antibody. The spectra therefore reflect TTR bound to RBP4. These results show that the occurrence of TTR isoforms is not affected by the binding of RBP4 to the TTR molecule and indicate that the TTR isoforms do not differ in their affinity for RBP4-binding. Therefore structural modifications of TTR molecule may not be involved in altered RBP4 metabolism in overweight T2DM subjects.

In summary, levels of RBP4 and TTR are not altered in overweight subjects and overweight subjects with T2DM with comparable kidney function. Moreover, factors stabilizing the RBP4-TTR interaction and thus influencing RBP4 retention such as the ratio of holo- and apo-RBP4 and RBP4 and TTR isoforms were not affected in these groups.

5.1.5 Effect of kidney dysfunction on RBP4 and TTR in humans

With regard to the relation of RBP4 in obesity and T2DM it is also of interest to study pathophysiological conditions which are known to be involved in RBP4 metabolism and in diabetes. Although RBP4 metabolism is related to kidney function, obesity and T2DM in turn might affect kidney function (Ritz 2008). In kidney dysfunction RBP4 levels increase. Since there were no differences in RBP4 and TTR levels in overweight and T2DM observed in this work, liver and kidney dysfunction which are known to interfere with RBP4 metabolism were investigated. Therefore RBP4 and TTR levels and isoforms were investigated in patients with CKD and after RTx.

Effect on RBP4 LEVELS

The elevation of RBP4 serum levels in CKD and ESRF has been described (Ziegelmeier *et al.* 2007; Frey *et al.* 2008), and is due to the decreased ability of the kidneys to filter low-molecular weight proteins, which in turn leads to an abnormal retention of small serum proteins, resulting in increased serum levels of these proteins (Vahlquist *et al.* 1973; Bernard *et al.* 1988). A loss of functional kidney tissue is associated with a disturbed RBP4 metabolism: It is assumed that in healthy subjects, apo-RBP4, after delivery of ROH to its target tissues, is glomerularly filtered and degraded subsequently to a megalin-mediated reabsorption in the proximal tubular system (Raila *et al.* 2005). Therefore, a decreased ability of the kidneys to filter RBP4 seems to be linked to an elevation of relative amounts of apo-RBP4 in serum, as supported by this study. The very low

eGFR in the CKD group may probably indicate that these patients have shrunken kidneys which are the result of renal ischemia (Ritz *et al.* 1999). It has already been demonstrated in acute renal failure in rats that this increase of apo-RBP4 seems to be a positive feedback signal for the hepatic release of ROH and RBP4, further increasing the RBP4 serum concentration (Gerlach and Zile 1990). The increase in RBP4 levels in CKD patients is also consistent with the results of Raila and Henze (2007). In this study RBP4 levels are increased in T2DM patients with normo- and microalbuminuria (an indicator of diabetic nephropathy), although eGFR was not different between the groups. RBP4 is also known to be a negative acute phase protein leading to reduced serum levels during specific diseases and inflammation. However, in T2DM patients with mild kidney dysfunction it could be shown that, despite increased Interleukin-6 levels, RBP4 remained increased (Raila *et al.* 2007). In addition, in a study with a large cohort of patients carried out by this research group it was shown that kidney function seems to influence RBP4 levels at the state of overt T2DM (Henze *et al.* 2008). Moreover, eGFR and uric acid were more important predictors of RBP4 levels than parameters such as LDL-cholesterol and triglycerides (Chang *et al.* 2008).

The significantly increased RBP4 levels in RTx patients compared to CKD patients point to the fact that glomerular filtration of RBP4 as well as reconstitution of RBP4 production, contribute to RBP4 serum levels (Kelleher *et al.* 1983). Previous studies indicated, that immunosuppressive therapies including corticosteroids may increase RBP4 serum levels (Borek *et al.* 1981). Increased RBP4 serum levels were also reported in patients after liver transplantation and have been contributed to the mobilization of stored ROH which was again secreted with RBP4 and TTR after transplantation due to the restored liver function (Mastroianni *et al.* 1998). Hence, in RTx patients, both, reduced RBP4 clearance due to impaired renal function as well as increased hepatic RPB4 production, may be responsible for increased RBP4 levels in RTx patients.

Effect on RBP4 ISOFORMS

In several studies isoforms of RBP4 and TTR have been discussed as potential factors that might influence the interaction of RBP4 and TTR (Jaconi *et al.* 1996; Frey *et al.* 2008; Mody *et al.* 2008). During renal disease RBP4 accumulates in serum due the decreased ability of the kidney to filter low-molecular weight proteins (Mogielnicki *et al.* 1971) and there is evidence for a truncation of RBP4 during CKD (Jaconi *et al.* 1995; Kiernan *et al.* 2002). In fact, in CKD and RTx

patients an elevation of specifically C-terminally truncated RBP4 isoforms was observed. It has been reported, that a specific carboxypeptidase truncates the RBP4 molecule in the serum of patients with severely deteriorated kidney function (Jaconi *et al.* 1996). Since RBP4 homeostasis is related to kidney function, the restoration of kidney function after RTx might also affect the occurrence of RBP4 isoforms. This is the first study that investigated RBP4 isoforms in patients undergoing RTx. In the study patients receiving a renal graft, the amounts of truncated RBP4 did not improve compared to HD patients. Since there is evidence for a specific carboxypeptidase truncating the RBP4 molecule in serum (Jaconi *et al.* 1996), the high amount of substrate (RBP4) may explain the increase in RBP4-L and RBP4-LL in RTx.

The kidneys play an important role in the recycling of RBP4 since RBP4 catabolism is disturbed in CKD patients (Smith and Goodman 1971; Kelleher *et al.* 1983). According to previous studies elevated serum creatinine levels, a marker for kidney dysfunction, are associated with high serum concentrations of RBP4 (Smith and Goodman 1971; Stewart and Fleming 1982). This is due to the loss of functional tissue and/or the entire nephron in kidney failure, which leads to decreased filtration of creatinine and abnormal survival of small serum proteins resulting in an increase of their serum levels (Waldmann *et al.* 1972; Vahlquist *et al.* 1973). This might explain the increased RBP4 levels in CKD (Table 2). Under physiological conditions 98% of RBP4 is bound to ROH (holo- RBP4) and 2% circulate ROH free as apo-RBP4.

Elevated RBP4 levels and insulin resistance and T2DM have been reported to be associated (Graham *et al.* 2006; Polonsky 2006) and RBP4 has been discussed as a marker for renal function in T2DM patients (Cabre *et al.* 2007; Takebayashi *et al.* 2007). Moreover, in a clinical study carried out by this institute serum RBP4 levels were increased in obese T2DM patients and were related to nephropathy despite inflammation and renal excretion of RBP4 (Raila *et al.* 2007). Within that context it is of interest that in this cohort correlations between systolic blood pressure, blood glucose, triglyceride and RBP4 levels were observed. These relations have been reported previously to be characteristic for the metabolic syndrome (Yoshida *et al.* 2006; Wolf 2007). Since there is a high prevalence of insulin resistance and T2DM in ESRF patients (Axelsson 2008) and in patients after RTx (Mannon 2008) compared to healthy subjects, future evaluation of RBP4 levels and RBP4 isoforms may provide information about the mechanism of how RBP4 may cause insulin

resistance. In fact, an elevation of apo-RBP4 during CKD but not in T2DM was shown here. The amount of apo-RBP4 has been discussed to be the mediator of insulin resistance and T2DM (Raila *et al.* 2007; Mills *et al.* 2008) since the correlations of the ROH/RBP4 ratio to diabetic risk factors were strong. Moreover, T2DM patients are exposed to increased oxidative stress which has been reported to be linked to endothelial dysfunction (Shurtz-Swirski *et al.* 2001). It is known that T2DM patients often suffer from kidney dysfunction (Whaley-Connell *et al.* 2008) and RBP4-L and RBP4-LL may further enhance oxidative stress through their action on polymorphonuclear leucocytes (Cohen and Horl 2004).

Effect on TTR LEVELS

TTR levels in CKD patients of this study were decreased compared to controls and RTx patients. TTR is known to be a nutritional marker as energy supply increases hepatic TTR synthesis (Cano *et al.* 1988). In addition, inflammation leads to decreased TTR levels. CKD patients in this study show high levels of CRP compared to controls and RTx indicating that liver synthesis of TTR may be strongly decreased in CKD patients due to inflammation. The reduction of TTR serum concentration is known for CKD patients and is mainly linked to the situation of malnutrition and inflammation during ESRF. The TTR levels in CKD patients were indeed below the cut-off value for possible protein-energy malnutrition and inflammation (Kopple *et al.* 2002). The fact that TTR levels in RTx patients were comparable to levels in healthy individuals is in agreement with other studies (Kelleher *et al.* 1983). However, high TTR levels were also reported in liver transplanted patients even one year after transplantation. The increase in TTR was related to an increase in RBP4 levels and was a marker for the restoration of the liver function (Mastroianni *et al.* 1998).

In patients with CKD, the levels of RBP4 were markedly elevated and therefore the molar ratio of RBP4 to TTR was increased. In healthy states, TTR is present in a 3-5 fold molar excess in plasma and the serum RBP4/TTR ratio is approximately 0.4 whereas in CKD patients an increase in the RBP4/TTR molar ratio up to 1.06 has been reported (Smith and Goodman 1971; Bernard *et al.* 1988; Cano *et al.* 1988; Cano 2002). This is consistent with the 3-fold elevated RBP4/TTR ratio from 0.36 in controls to 0.96 in CKD in this study. Due to the increase of RBP4 and the simultaneous fall in TTR levels in CKD, RBP4 and TTR are present in a 1:1 stoichiometry in the circulation of these patients (Smith and Goodman 1971; Kelleher *et al.* 1983; Bernard *et al.* 1988). The decrease in TTR levels in CKD may

be due to malnutrition and/or infectious disease (Smith and Goodman 1971; Cano *et al.* 1988).

Effect on TTR ISOFORMS

Although TTR metabolism is hardly affected by kidney failure, the data in CKD and RTx patients show modifications in the TTR molecule. The spectra of CKD and RTx patients display significantly higher amounts of cysteinylated, cysteinylglycinylated and glutathionylated TTR in relation to native TTR compared to controls. During kidney disease oxidative stress is increased in HD patients mainly due to the activation of polymorphonuclear neutrophil leukocytes during dialysis which in turn may release cytokines and induce inflammation (Sommerburg *et al.* 1999). The observed TTR isoforms may therefore be the result of a reduced antioxidative capacity in CKD. In addition, the strong correlation of TTR isoforms and eGFR supports the relation of an altered radical metabolism due to reduced kidney function. To that date TTR which is a stable plasma protein in healthy states, is known to be involved in TTR-related amyloidosis. The disease may lead through the generation of fibrils to organ dysfunction. Thus the occurrence of TTR isoforms in CKD and RTx may be of interest since organ complications after RTx have been reported in amyloidosis patients (Haq *et al.* 2007).

In conclusion, this study provides new insights on RBP4 and its isoforms in patients with CKD and after RTx. Besides elevated RBP4 levels during CKD and after RTx it could be demonstrated that apo-RBP4 and two specific C-terminally truncated RBP4 isoforms, RBP4-L and RBP4-LL, seem to be strongly related to kidney function. With regard to TTR, the levels were decreased in CKD due to dialysis and were increased after RTx compared to control. Interestingly, the relative amount of cys-, cysglyc- and glut-TTR was increased in CKD and RTx compared to controls.

5.1.6 Effect of liver dysfunction on RBP4 and TTR in humans

This part of the study was designed to investigate the effect of CLD on RBP4 and TTR levels and isoforms. The results show that RBP4 and TTR levels were significantly decreased in serum of CLD patients compared to controls. Apo- and holo-RBP4 as well as RBP4-L and RBP4-LL were not different in patients with CLD. Interestingly, TTR isoforms, i.e. the relative amounts of the cysteinylated and glutathionylated forms of native TTR, were significantly increased in CLD patients as compared to controls.

Effect on RBP4 LEVELS

Repeated hepatic injury leads to the development of liver diseases such as fibrosis which progressively worsens to cirrhosis and hepatic cancer. Interestingly, this process is accompanied by the loss of ROH containing lipid droplets of stellate cells. The reduced ROH levels in CLD patients have been reported not to be the result of malnutrition but rather the enhanced mobilization of ROH to peripheral tissues (Blaner *et al.* 2008). In this study, RBP4 levels were markedly depressed in the serum of patients suffering from various liver diseases, which is in accordance with results of previously published studies (Smith and Goodman 1971; Newsome *et al.* 2000; Yuan *et al.* 2006; Bahr *et al.* 2008). This decrease is due to a loss of functional hepatic tissue resulting in decreased synthesis of RBP4 and TTR and decreased release of the ROH-transport complex into the circulation (Ingenbleek *et al.* 1975; Newsome *et al.* 2000). Moreover, the percentage of plasma apo-RBP4 was not altered in CLD patients compared to controls. Since, nearly all of the apo-RBP4 is normally glomerularly filtered and reabsorbed by the kidney proximal convoluted tubules the amount of apo-RBP4 is serum of subjects with normal kidney function is low (Peterson and Berggard 1971; Kelleher *et al.* 1983; Jaconi *et al.* 1996; Willnow *et al.* 1996). This finding is confirmed by the correlation of apo-RBP4 and serum creatinine in the study in this work.

Effect on RBP4 ISOFORMS

RBP4-L and RBP4-LL were not altered in patients with various CLD. Since the increase in RBP4 isoforms was not observed in patients suffering from various CLD, suffering from overweight and T2DM, the important physiological function of the kidneys in that context is emphasised. This is underlined by the fact that RBP4 isoforms were highly increased in CKD and RTx patients. The impaired catabolism of RBP4 in the kidneys may thus lead to an accumulation of RBP4 isoforms in serum. Therefore the results support the hypothesis that the C-terminal truncation of RBP4 may be specific during CKD.

Jaconi et al. (Jaconi *et al.* 1996) investigated RBP4-L and RBP4-LL in the serum of HD patients and regarded the occurrence of RBP4 isoforms to be specific for CKD. Apart from the above mentioned study of RBP4 and TTR isoforms in CKD and RTx, to date RBP4 isoforms have been investigated exclusively in a small number of patients (1 and 10, respectively) suffering from CKD (Jaconi *et al.* 1995; Kiernan *et al.* 2002) and not in CLD patients. The data of this work show for the first time that

RBP4-L and RBP4-LL, which are truncated at the C-terminal end of the molecule, are not altered in patients suffering from CLD.

To further investigate, RBP4 levels and isoforms with regard to specific CLD, the CLD patients were divided according to the diagnosis of the liver disease (fibrosis, HCC, NAFLD). RBP4 levels were decreased in patients with fibrosis, HCC and NAFLD compared to control. Since the kidney function evaluated by the eGFR was not different between control subjects and patients suffering from fibrosis, HCC and NAFLD, the reduction in RBP4 levels seems to be the result of the liver dysfunction. Decreased RBP4 levels in cirrhosis have been reported and due to the loss of functional tissue. During cirrhosis an association of RBP4 levels and insulin resistance has been reported. It has thus been postulated that liver function has to be considered when interpreting RBP4 levels (Bahr *et al.* 2008).

Interestingly, NAFLD showed a different pattern of RBP4 levels and isoforms: RBP4 levels in NAFLD patients are increased compared to HCC and fibrosis patients and decreased compared to controls. Independent of the incidence of T2DM, patients suffering from NAFLD have been reported to have higher RBP4 levels than patients with other CLD (Seo *et al.* 2007; Wu *et al.* 2007). Moreover, NAFLD patients have higher ALT levels due to the injured hepatocytes. Therefore as the liver is the main site of RBP4 synthesis, hepatocyte damage may lead to a decrease in RBP4 serum levels (Wu *et al.* 2007). An increased amount of visceral fat distribution is associated with an increased risk of insulin resistance and therefore a more aggressive form of NAFLD and increased RBP4 levels were positively associated with elevated liver fat in healthy subjects (Kang *et al.* 2006). With regard to the RBP4 truncation, it was shown that RBP4-L and RBP4-LL are not present in HCC and fibrosis patients. NAFLD patients, however, exhibit increased amounts of RBP4-L compared to controls and the other groups. The reason for that has to be elucidated. However, the pathogenesis process of NAFLD to hepatic fibrosis and cirrhosis include stellate activation and oxidative stress. Thus the disease may have impact on RBP4 metabolism e.g. by the loss of ROH droplets during stellate activation (Farrell and Larter 2006).

Effect on TTR LEVELS

TTR levels were decreased in patients with CLD which is consistent with results from previously published studies: Due to the decreased capability of the liver to synthesize proteins the synthesis of TTR is also affected since its synthesis is

located in the liver (Bernstein and Ingenbleek 2002). It has been reported that TTR is regarded – among 21 different plasma proteins – to be the most sensitive indicator of hepatic malfunction, since its levels are negatively correlated with the extent of liver damage (Ingenbleek *et al.* 1975). Therefore the dysfunction of the liver leads to a decrease in serum TTR levels (Smith and Goodman 1971; Newsome *et al.* 2000). The lower values for TTR (as well as for RBP4) in CLD patients are at least in part the result of a faster turnover rate. In fact, the biological half-life of albumin is about 20 days whereas the half-life of TTR is estimated about 2 days (Ingenbleek *et al.* 1975). Moreover, due to inflammation in CLD patients – indicated by higher CRP levels compared to controls – TTR secretion may additionally be subject to a decreased synthesis (Abraham *et al.* 2003). Interestingly, there is an increase in TTR levels in patients after liver transplantation which underlines the importance of a good liver function for TTR secretion (Mastroianni *et al.* 1998).

Effect on TTR ISOFORMS

In CLD patients the relative amounts of cys-TTR and glut-TTR were increased compared to controls. To that date TTR isoforms have not yet been investigated in patients suffering from various CLD. In fact, TTR isoforms are only known to be a direct potential risk factor for the development of amyloidosis (Kingsbury *et al.* 2008) and to be a marker for ovarian cancer (Moore *et al.* 2006). Moreover, increased TTR isoforms were also reported in multiple trauma patients and were related to antioxidative status (Gericke *et al.* 2007). Therefore the increase in TTR isoforms in CLD patients may be related to the inflammatory status which is associated with an increase in oxidative stress. In particular, during infection an activation of inflammatory cells including neutrophils and macrophages leads to the production of inflammatory mediators such as reactive oxygen species. These mediators, in turn, increase levels of oxidants and decrease levels of antioxidants creating oxidative stress (Ernst 1999). In a publication by this institute it could be shown that, oxidative stress in rats did alter the TTR spectra and thus the occurrence of TTR isoforms (Henze *et al.* 2008). However, due to the ability of the TTR molecule to form a mixed disulfide with its cysteine residue at position 10 and, for instance, glutathione, it might therefore protect TTR from oxidative damage (Gericke *et al.* 2007).

In conclusion, during liver dysfunction the synthesis of RBP4 and TTR is affected leading to a reduction in RBP4 and TTR serum levels. Moreover RBP4 isoforms were

not affected by reduced liver function whereas TTR isoforms during liver dysfunction were increased and may indicate oxidative stress.

5.2 Cellular retinol-binding protein type I (CRBP-I)

Retinoids are known to activate the transcription of over 500 genes and it has been postulated that retinoids are new players in the development of obesity and T2DM through their action on glucose metabolism and adipose tissue physiology (Corbetta *et al.* 2006; Desvergne 2007). The adipose tissue is – next to the liver – a major storage site for retinoids. To that date knowledge on the extra-hepatic metabolism of ROH and on intracellular retinol-binding protein CBRP-I is limited. It is known that CRBP-I is needed for ROH esterification and storage in the liver (Ghyselinck *et al.* 1999). Therefore the aim of the second part of this work was to investigate the functions of CRBP-I in extra-hepatic tissues.

5.2.1 Absence of CRBP-I in the liver

CRBP-I is known to mediate the intracellular esterification of ROH to RE within the liver. In fact, under a regular diet the hepatic RE stores were lower in CRBP-I KO mice compared to WT mice. LRAT, the major ROH esterifying enzyme in the liver, leads to the formation of RE from ROH supplied by CRBP-I. It has been reported that the decrease in hepatic RE levels in CRBP-I KO mice is due to an impaired delivery of ROH to LRAT. This is consistent with the results of this experiment indicating that LRAT seems to be exclusively capable of esterifying ROH delivered by CRBP-I in the liver. This is underlined by the unchanged hepatic expression levels of LRAT in CRBP-I KO mice here and in previous studies (Ghyselinck *et al.* 1999; Noy 2000). Moreover, ROH bound to CRBP-I is the substrate for both, the enzymatic esterification by LRAT and oxidation to RA by ADH1 (Okuno *et al.* 1995). The function of LRAT is thus to channel ROH away from its oxidative activation to RA (Kim *et al.* 2008) since intracellular RA has to be maintained at a low but steady level (Napoli 1999). However, ADH1 has been reported to continuously oxidize ROH to RA independent of dietary ROH conditions. Interestingly, in mice with the genetic deletion of both, CRBP-I and ADH1, hepatic RE stores were protected (Molotkov *et al.* 2004) indicating mechanisms for RE synthesis apart from LRAT. Therefore one might assume that in the absence of CRBP-I ROH oxidation increases due to the (unchanged) activity of ADH1 which has access to a higher amount of substrate. The decreased RE levels in CRBP-I KO mice might also be the result of a decreased uptake of ROH into the liver cells. It has been reported that cellular uptake is

dependent on the presence of CRBP-I (Napoli 1999) and indeed in CRBP-I KO mice the ROH levels in the liver were lower compared to the WT mice (Table 10 at page 57).

Moreover, the lack of CRBP-I and the related decrease in hepatic RE stores, makes CRBP-I KO mice susceptible for developing rapid retinoid deficiency (Ghyselinck *et al.* 1999). This is in accordance with the results in this experiment: CRBP-I KO mice show depleted hepatic RE stores after 8 weeks on a vitamin A insufficient diet compared to WT mice.

5.2.2 Function of CRBP-I in adipose tissue

Apart from the knowledge that the adipose tissue is the second largest retinoid storage site of the body, data on ROH metabolism remain limited. Interestingly, ROH and RE levels were higher in epididymal fat pads of CRBP-I KO mice compared to WT mice although serum ROH levels remained unchanged among the genotypes. Even after 8 weeks on a vitamin A insufficient diet ROH levels are higher in CRBP-I KO mice and the depletion of RE stores is slowed down in the absence of CRBP-I compared to WT mice. This indicates that in the absence of CRBP-I and even under dietary hypovitaminosis A specific mechanisms must be induced to maintain RE levels. One might speculate that a higher amount of ROH is transported to the adipocyte in CRBP-I KO mice triggered by the decreased capability of the liver to take up ROH. However, postprandial [^{3}H]-retinoid uptake (reflecting short-term uptake within 24 hours) in adipose tissues was not altered in CRBP-I KO mice compared to WT mice. Therefore mechanisms in the cell seem to be responsible for the increased ROH and RE levels in adipose tissue of CRBP-I KO mice.

Interestingly, mRNA levels of *STRA6,* the cell surface receptor for RBP4, in adipose tissue of CRBP-I KO mice was higher indicating that the uptake of ROH into adipocytes might be facilitated. STRA6 has recently been identified as a membrane receptor for ROH bound to RBP4 and mediates the ROH uptake into the cells (Blaner 2007). Therefore long-term uptake of ROH into the cells might be facilitated in CRBP-I KO mice. Moreover, in the cell ROH can be bound to CRBP-I or in certain tissues such as the adipose tissue to CRBP-III. Due to the simultaneous elevation of *CRBP-III* mRNA levels in CRBP-I KO mice compared to WT mice, one might assume that STRA6 enhances ROH uptake into the cell and the further binding to CRBP-III might lead to the higher ROH levels in CRBP-I KO mice. An increase in CRBP-III

levels has also been reported in the adipose tissue of LRAT KO mice (O'Byrne *et al.* 2005).

Interestingly, CRBP-III is expressed in adipocytes and stromal vascular cells of the adipose tissue, whereas CRBP-I is almost exclusively expressed in stromal vascular cells. Stromal vascular cells contain preadipocytes, endothelial cells and macrophages. The latter ones are involved in adipokine secretion in obesity and T2DM (Guilherme *et al.* 2008). The function of CRBP-I in stromal vascular cells remains unclear but implies that WAT is a target tissue for retinoid action (Tsutsumi *et al.* 1992). Therefore, the absence of CRBP-I may change retinoid action in adipose tissue. Moreover, there is evidence that CRBP-I and CRBP-III work together, since in CRBP-III KO mice CRBP-I expression is increased in heart, adipose tissue and mammary gland (Vogel *et al.* 2001). On the other hand, CRBP-III expression is induced in the adipose tissue of CRBP-I KO mice (Piantedosi *et al.* 2005). The absence of one of the two proteins results in the up-regulation of the other protein and both, CRBP-I and CRBP-III contribute to the regulatory network of ROH metabolism within the cell (Piantedosi *et al.* 2005)

Increased STRA6 and CRBP-III expression explains the higher ROH levels in adipose tissue of CRBP-I KO mice but not the higher RE levels. Within the cell, ROH can be esterified by LRAT (or the enzymatic activity ARAT) or it can be oxidised by ADH1 and RALDH. In mice lacking the ROH esterifying enzyme LRAT, ROH and RE levels have been reported to be elevated in adipose tissue (Kim *et al.* 2008). The results of this work and others indicate that in the absence of the liver to synthesize RE, the adipose tissue becomes a major storage site for retinoids. In fact, the adipocytes of the WAT have the second largest retinoid stores of the body (the largest retinoid levels are found in the hepatic stellate cells) (Wei *et al.* 1997). However, mRNA levels of retinoid metabolizing enzymes such as LRAT, ADH1 and RALDH in the adipose tissue were not altered in CRBP-I KO mice. This made one speculate that there have to be other enzymes being capable to esterify ROH. Indeed, to that date the enzymes carrying out the ARAT reaction are still under investigation. However there is evidence that enzymes involved in triglyceride synthesis such as members of the MGAT and DGAT family may be capable to esterify ROH. Interestingly, ROH esterification is indeed almost inseparable from triacylglycerol synthesis: Both, ROH and 2-monoacylglycerols are packed into chylomicrons and transported to the liver after intestinal absorption (Orland *et al.* 2005). The isoforms MGAT1, MGAT2 and DGAT1 are expressed in several tissues

including adipose tissue. There is evidence that these members of the MGAT and DGAT family are capable of carrying out the ARAT reaction (Orland *et al.* 2005; Shi and Cheng 2008). In fact, it was shown in this work in HEK293 cells, over expressing human MGAT1, MGAT2 and DGAT1, that MGAT1 and DGAT1 were able to catalyse the esterification of ROH to RE.

DGAT1 mRNA could also be detected in adipose tissue of CRBP-I KO mice. Thus DGAT1 may be able to carry out the ARAT reaction by esterifying ROH and increase RE storage in CRBP-I KO mice. Interestingly, DGAT1 is mostly expressed in the adipose tissue (Yen *et al.* 2005) and uses free ROH and less well CRBP-III bound ROH as a substrate. This is in accordance with the fact that ARAT activity is dependent on coenzyme A-activated fatty acids as direct acyl donors (Orland *et al.* 2005) and DGAT1 does not use ROH bound to CBRP-I (O'Byrne *et al.* 2005). Therefore its activity is independent of the absence or presence of CRBP-I. In the presence of CRBP-I the RE synthesis by DGAT1 is suppressed due to the decreased amount of free ROH. Interestingly, LRAT has a significantly lower K_m for ROH than ARAT (2 µM and 15 µM, respectively) (Yen *et al.* 2005). Therefore, the high RE levels in adipose tissue of CRBP-I KO mice may be the result of an increased activity of the ARAT reaction. In fact, in DGAT1 KO mice maintained on a HFD the hepatic RE levels were increased whereas to that date changes in adipose tissue in DGAT1 KO mice have not been investigated (Yen *et al.* 2005). Thus, it might be possible that in the absence of CBRP-I and a reduced transport of ROH to LRAT, RE levels in extra-hepatic tissues may increase due to an increased ARAT activity. These and previously reported data indicate that RE storage and metabolism are different in adipose tissue and the liver (O'Byrne *et al.* 2005).

5.2.3 Function of CRBP-I in the pancreas

Data on retinoid metabolism in the pancreas is very limited, although it has been reported that RBP4, TTR and CRBP-I are expressed in high amounts in the islets of Langerhans in the pancreas of rats compared to the amounts in the liver (Kato *et al.* 1985). Moreover, these authors found high levels of RBP4, TTR and CRBP-I in isolated rat islets and the cellular localization of CRBP-I resembled to that of glucagon. Retinoids are also known to influence insulin (Chertow *et al.* 1983) and glucagon (Chertow *et al.* 1987) release from islets and thus it was speculated that retinoids and their binding proteins may be involved in the endocrine function of the pancreas (Kato *et al.* 1985). In this experiment it was shown that under a regular

diet RE levels in the pancreas were decreased in CRBP-I KO mice whereas ROH levels were slightly but not significant lower. However, decrease in RE levels did not increase ROH oxidation pathways, since mRNA levels of ADH1 and RALDH were not altered in pancreas. In the above mentioned study CRBP-I levels in the islets have been reported to be higher than levels observed in other rat tissues (Kato *et al.* 1985). Therefore, in the absence of CRBP-I ROH uptake into the pancreatic cells may be decreased compared to WT mice. This might thus also affect insulin secretion and turnover. Interestingly, after 8 weeks on vitamin A insufficient diet neither ROH nor RE levels in the pancreas of CRBP-I KO mice were different from levels in WT mice although a decrease would be expected in the absence of CRBP-I. Therefore it seems reasonable that in the pancreas there must be a protection of RE stores in times of hypovitaminosis.

Since it is known that retinoids are needed for insulin secretion, the lack of CRBP-I in CRBP-I KO mice might also lead to a decreased ROH mobilization within the pancreatic cells and a less active insulin secretion. Therefore the pancreas might be dependent of newly absorbed ROH. Interestingly, postprandial retinoid absorption showed that the total amount of [^{3}H]-retinoids entering the pancreas is comparable to the amount which enters the epididymal fat. Moreover, after 2, 6 and 24 hours [^{3}H]-retinoid levels were higher in CRBP-I KO mice compared to WT mice. This interesting fact makes one speculate that in the absence of CRBP-I, when ROH cannot be efficiently stored in the pancreas, a higher amount of ROH is taken up into the pancreas to assure insulin secretion. These results further underline the importance of retinoids in the pancreas.

5.2.4 Function of CRBP-I in the lungs

Next to the liver and adipose tissue the lungs also store significant amounts of ROH as REs and lipid containing droplet stellate cells were identified in lung tissue (Nagy *et al.* 1997). The reason for the localization of stellate cells in the lung is due to the fact that lung development, maturation and maintenance of matured lungs is dependent on retinoids (Ross *et al.* 2006). The local storage of RE makes the fetal lung independent of the liver vitamin A supply. It has also been shown that the lungs contain RA-binding proteins and express several isoforms of nuclear retinoic acid receptors. This is further underlined by the fact that human neonates showing ROH deficiency suffer from respiratory problems (Chytil 1992). It has been reported that CRBP-I is highly expressed in the lungs whereas CRBP-III is not (Vogel *et al.*

2001). Lower ROH and RE levels have been reported in CRBP-I KO foetuses whereas later in life the levels were similar (Ghyselinck *et al.* 1999). Conversely, in the CRBP-I KO mice in this work the RE stores were higher compared to the WT mice although ROH binding to CRBP-III can not account for that. The mechanisms for the higher lung content have to be elucidated in further studies.

5.2.5 Effect of the absence of CRBP-I on body composition

The CRBP-I KO mice in this work develop normally and show no obvious phenotypic differences compared to WT mice and when maintained on a ROH sufficient diet. This is in accordance with the data evaluated by Ghyselinck and Bavik (1999) who reported that CRBP-I KO mice are healthy and fertile when fed a ROH containing diet. However, it could be shown in this work that CRBP-I KO mice gain less weight later in life compared to WT mice although relative amount of body fat is not different between the genotypes. This could be contributed to RA, the active metabolite of ROH, which regulates cell proliferation and differentiation (Sporn *et al.* 1994). Interestingly, RA deprivation may lead to weight loss (Ghyselinck *et al.* 1999). Since CRBP-I is involved in ROH oxidation to RA by transporting ROH to the metabolizing enzymes, ADH1 and RALDH, the absence of CRBP-I might lead to a decrease in RA synthesis and thus body weight gain later in life (Okuno *et al.* 1995). However, RA levels were not investigated in this work.

In several works it has been shown that certain proteins involved in glucose metabolism are regulated by retinoids (Blumentrath *et al.* 2001). Moreover, insulin secretion is also dependent on the availability of vitamin A. Since decreased retinoid levels were reported in the pancreas of CRBP-I KO mice, the aim was to evaluate insulin sensitivity in CRBP-I KO mice by means of an insulin tolerance test. The test is used to analyse peripheral glucose uptake. It has been shown in this work, although serum glucose levels were not altered after 6 hours fasting in CRBP-I KO mice compared to WT mice but there was a decreased uptake of glucose 90 minutes after insulin administration in CRBP-I KO mice. This has not been investigated to that date and the interpretation of these results is thus difficult:
There is evidence that the oral intake of RA does enhance glucose production in hepatocytes and insulin release from pancreatic β-cells (Corbetta *et al.* 2006). Thus a decrease of RA levels might affect insulin sensitivity and be the result of the disturbed transport of ROH to its oxidizing enzymes in CRBP-I KO mice (Molotkov *et al.* 2004). Since RA is needed for insulin secretion a limited amount of RA in CRBP-I

KO mice might increase insulin turnover and thus glucose levels. However, RA levels have not been measured in the mice of this work. In addition, RA has been reported to increase glucokinase activity in the liver and thus insulin secretion in the pancreas. Therefore alterations in RA synthesis might disturb normal insulin homeostasis (Cabrera-Valladares *et al.* 1999). Within that context, it is interesting that in mice with the genetic deletion of the enzyme RALDH*,* the endogenous RAL levels increased and protected the mice from insulin resistance in diet-induced obesity (Ziouzenkova *et al.* 2007). Therefore, in CRBP-I KO mice an increase of intracellular RAL levels might also affect insulin and glucose metabolism.

There have been several studies reporting that retinoids may also influence lipid metabolism (Koistinen *et al.* 2001; Corbetta *et al.* 2006). In this work, the results of serum lipid levels were conflicting: At the age of 21 weeks free fatty acid levels were higher in WT mice but not at an older age of 37 weeks compared to CRBP-I KO mice. Moreover, at the younger age of 21 weeks triglycerides were not different between CRBP-I KO and WT mice but were higher later in life in WT mice.

It has been reported that the oral intake of 13-cis-RA in men did alter their serum lipid profile dependent on the duration of the intake leading to an increase in triglyceride levels along with a decrease in HDL cholesterol (Koistinen *et al.* 2001; Corbetta *et al.* 2006). Moreover, treatment of adipocytes with RA did increase oxidative metabolism and decrease triglyceride levels in adipocytes (Mercader *et al.* 2008). Therefore the altered ROH metabolism probably leading to alterations in RA synthesis might affect triglyceride levels in CRBP-I KO mice. The interpretation of the results on serum lipids is limited due to the fact that RAL and RA levels were not measured in the investigated animals.

Within the context of serum lipids the functions of CRBP-III in the adipose tissue are of interest, since in CRBP-I KO mice the CRBP-III expression in adipose tissue was increased. In a recent publication CRBP-III KO mice were shown to have reduced serum free fatty acid levels and fatty liver in diet-induced obesity although there was no difference in weight gain (Zizola *et al.* 2008). In the absence of CRBP-III in mice there is a decreased efflux of glycerol and free fatty acids from adipocytes thus protecting the liver of steatosis (Zizola *et al.* 2008). However, to that date, there are no data published on serum lipids in CRBP-I KO mice.

6 Summary

The fat-soluble vitamin A, which is chemically referred to retinol (ROH), is known to be essential for the process of vision, the immune system but also for cell differentiation and proliferation. Recently, ROH itself has been reported to be involved in adipogenesis and a ROH transport protein, the retinol-binding protein 4 (RBP4), in insulin resistance and type 2 diabetes. However, there is still considerable scientific debate about this relation. With the increasing amount of studies investigating the relation of ROH in obesity and type 2 diabetes, basic research is an essential prerequisite for interpreting these results. This thesis enhances the knowledge on this relation by reviewing ROH metabolism on extra- and intracellular level.

Aim 1: In the blood stream ROH is transported in a complex with RBP4 and a second protein, transthyretin (TTR), to the target cells. The levels of RBP4 and TTR are influenced by several factors but mainly by liver and kidney function. The reason for that is that liver and the kidneys are the sites of RBP4 synthesis and catabolism, respectively. Interestingly, obesity and type 2 diabetes involve disorders of the liver and the kidneys. Therefore the aim was to investigate factors that influence RBP4 and TTR levels in relation to obesity and type 2 diabetes (Part 1).

Aim 2: Once arrived in the target cell ROH is bound to cellular retinol-binding protein type I (CRBP-I) and metabolised: ROH can either be stored as retinylesters or it can be oxidised to retinoic acid (RA). By acting as a transcription factor in the nucleus RA may influence processes such as adipogenesis. Therefore vitamin A has been postulated to be involved in obesity and type 2 diabetes. CRBP-I is known to mediate the storage of ROH in the liver, but the extra-hepatic metabolism and the functions of CRBP-I are not well known. This has been investigated in Part 2 of this work.

Material & Methods: RBP4 and TTR levels were investigated by ELISA in serum samples of human subjects with overweight, type 2 diabetes, kidney or liver dysfunction. Molecular alterations of the RBP4 and TTR protein structure were analysed by MALDI-TOF mass spectrometry. The functions of intracellular CRBP-I were investigated in CRBP-I knock-out mice in liver and extra-hepatic tissues by

measuring ROH levels as well as the levels of its storage form, the retinylesters, using reverse phase HPLC. The postprandial uptake of ROH into tissues was analysed using labelled ROH. The mRNA levels of enzymes that metabolize ROH were examined by real-time polymerase chain reaction (RCR).

Results: The previous published results showing increased RBP4 levels in type 2 diabetic patients could not be confirmed in this work. However, it could be shown that during kidney dysfunction RBP4 levels are increased and that RBP4 and TTR levels are decreased during liver dysfunction. The important new finding of this work is that increased RBP4 levels in type 2 diabetic mice were increased when kidney function was decreased. Thus an increase in RBP4 levels in type 2 diabetes may be the effect of a reduced kidney function which is common in type 2 diabetes. Interestingly, during severe kidney dysfunction the molecular structure of RBP4 and TTR was altered in a specific manner which was not the case during liver diseases and type 2 diabetes. This underlines the important function of the kidneys in RBP4 metabolism.

CRBP-I has been confirmed to be responsible for the ROH storage in the liver since CRBP-I knock-out mice had decreased ROH and retinylesters (the storage form of ROH) levels in the liver. Interestingly, in the adipose tissue (the second largest ROH storage tissue in the body) ROH and retinylesters levels were higher in the CRBP-I knock-out compared to the wild-type mice. It could be shown in this work that a different ROH binding protein, cellular retinol-binding protein type III, is upregulated in CRBP-I knock-out mice. Moreover enzymes were identified which mediate very efficiently ROH esterification in the adipose tissue of the knock-out mice. In the pancreas there was a higher postprandial ROH uptake in the CRBP-I knock-out compard to wild-type mice. Even under a vitamin A deficient diet the knock-out animals had ROH and retinylesters levels which were comparable to wild-type animals. These results underline the important role of ROH for insulin secretion in the pancreas.

Summing up, there is evidence that RBP4 levels are more determined by kidney function than by type 2 diabetes and that specific molecular modifications occur during kidney dysfunction. The results in adipose tissue and pancreas of CRBP-I knock-out mice support the hypothesis that ROH plays an important role in glucose and lipid metabolism.

7 Literature

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8 Supplemental Material

Primer sequences

LRAT: 5’-gcagttgggactgactccat-3’, 5’-cagattgcaggaagggtca-3’

ADH1: 5’-acaaacccttcaccatcgag-3’, 5’-ccttctccaacgctctcaac-3’

RALDH1: 5’-tttggccacacactccaata-3’, 5’-gggctgacaagattcatggt-3’

STRA6: 5’-ctgttggaccagatgctgaa-3’, 5’-gcaacagagtggaggaggag-3’

CRBP-III: 5’-caggctctctggaaggtttg-3’, 5’-aaatgcacgagcctggttac-3’

humMGAT1: 5’-ccagcggaaaggatttgtta-3’, 5’-caaagcaaaccccatgatct-3’

hummGAT1: 5’-agagcacaggcttctcttcg-3’, 5’-aatgatgcccagcaagtttc-3’

humDGAT I: 5’-ggcctgccccatgcgtgattat-3’, 5’-ccccactgaccttcttccctgtaga-3’

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"Wir sind nicht lose, unabhängige und für sich bestehende Einzelwesen, sondern wie Glieder in einer Kette, und wir wären, so wie wir sind, nicht denkbar ohne die Reihe derjenigen, die uns vorangingen und uns die Wege wiesen."

[Thomas Mann: Buddenbrooks: The decline of a family]

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10 Statutory Declaration

I hereby declare that this thesis has been written by myself without any external unauthorised help, that it has been neither presented to any institution for evaluation nor previously published in its entirety or in parts. Any parts, words or ideas, of the thesis, however limited, and including tables, graphs, maps etc., which are quoted from or based on other sources have been acknowledged a as such without exception.

Berlin, 29 May 2009

11 Curriculum vitae

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UNIVERSITY EDUCATION

06/2005	**University of Vienna, Austria** "Magister rer. nat." in Nutrition Advisor: Prof. Dr. I. Elmadfa; Grade: Very Good
07/2004 – 04/2005	**University of Potsdam, Germany** Diploma Thesis: „Salivary biomarker of diabetic persons (type I)". Advisor: Prof. Dr. F. J. Schweigert; Grade: Excellent
03/2002 – 04/2004	**University of Vienna, Austria** (Advanced study period)
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WORK EXPERIENCE

10/2005 – 02/2009	**Institute for Physiology and Pathophysiology of Nutrition University of Potsdam, Germany** Ph.D. Student, Graduate Research Assistant **Society of applied Vitamin Research (GVF)** Director branch office
04/2008 - 10/2008	**Department of Preventive Medicine and Nutrition, Columbia University, New York, USA** Vogel Group, Scientific assistant Scholarship of the German Academic exchange Service (DAAD)
10/2004 - 12/2004	**Konsument, Stiftung Warentest, Vienna, Austria** Content manager of the journal „90 Diet Programs Tested" (publication date: May 2005)
01/2002 - 02/2002	**Stiftung Warentest, Berlin, Germany** Internship, Division: Product testing and conception
10/2001 - 12/2001	**Consumer Advice Center Hessen, Frankfurt a. M., Germany** Internship, Division: Nutrition
09/1999 - 10/1999	**Clinical Center Augsburg, Germany** Internship, Division: Composition of specific Diets
01/1999 - 07/1999	Au-pair-residence in Marseille, France

12 JOURNAL PUBLICATIONS

(1) **"Alterations of Retinol-binding protein 4 isoforms in patients with chronic kidney disease and their relation to lipid parameters"**
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Frey SK, Spranger J, Henze A, Pfeiffer AF, Schweigert FJ., Raila J. *Metabolism* 2009 (accepted).

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