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Anne Kunnari

GENETIC, EPIDEMIOLOGICAL AND CELL CULTURE STUDIES ON HUMAN RESISTIN

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ANNE KUNNARI

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Academic dissertation to be presented, with the assent of the Faculty of Medicine of the University of Oulu, for public defence in Auditorium 8 of Oulu University Hospital, on December 12th, 2008, at 12 noon

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Abstract

Resistin was discovered in the year 2000. In the mouse, it was reported to be produced by adipocytes and to be linked with insulin resistance and obesity. Human resistin has been shown to be produced by leucocytes but the literature contains contradictory results on its association with obesity and type 2 diabetes. Aim of this study was to clarify the role of resistin in the human context especially in type 2 diabetes and atherosclerosis.

The first study examined the possible association of human resistin gene variants with type 2 diabetes. The studied three variants were not associated with type 2 diabetes though they were demonstrated in the second study to be associated with the plasma resistin concentration. However, two gene variants were associated with the prevalence of cerebrovascular disease in subjects with type 2 diabetes.

In the third work, the association of the plasma resistin level with the risk factors of atherosclerosis and early atherosclerosis measured with carotid artery intima-media thickness (IMT) were studied. Plasma resistin level was not associated with IMT independently from the known risk factors of atherosclerosis. However, resistin was associated with inflammatory markers highly sensitive CRP and the number of leucocytes whereas insulin resistance did not associate with resistin.

In the fourth study, resistin expression in different leucocytes and its modulation, as well as the effect of resistin on monocyte adhesion to endothelium were evaluated. The novel discovery was that resistin is expressed in all the main leucocyte lineages, with monocytes and neutrophils exhibiting the highest relative mRNA and protein levels of resistin. Resistin expression was up-regulated by pro-inflammatory factors in the cells of both innate and adaptive immunity. The present results demonstrating that resistin increases adhesion and expression of some adhesion molecules support the hypothesis that resistin may be involved in the development of atherosclerosis.

The above results indicate that resistin is widely produced by leucocytes and therefore may participate in inflammatory processes. Since it may be considered as an inflammatory cytokine, resistin may also influence the development of atherosclerosis. In the future, resistin could possibly be used as a marker of inflammation.

Keywords: atherosclerosis, inflammation, leucocyte, polymorphism, resistin, type 2 diabetes

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Tiivistelmä

Vereen erittyvä uusi hormoni, resistiini, löydettiin vuonna 2000. Hiirellä resistiinin on havaittu erittyvän rasvasoluista ja sen on arveltu toimivan linkkinä lihavuuden ja insuliiniresistenssin välillä. Ihmisellä resistiinin tehtävä on toistaiseksi huomattavasti epäselvempi ja toisin kuin hiirellä ihmisen resistiinin korkein ilmentymistaso on valkosoluissa. Tämän väitöstutkimuksen tarkoituksena oli selvittää ihmisen resistiinin toimintaa ja erityisesti sen liittymäkohtia tyypin 2 diabetekseen ja ateroskleroosiin.

Ensimmäisessä osatyössä on selvitetty resistiini-geenin nukleotidimuuntelun yhteyttä tyypin 2 diabetekseen ja siihen liittyviin tekijöihin diabetespotilasaineistossa. Resistiinin geenimuuntelu ei tulosten perusteella ole yhteydessä tyypin 2 diabetekseen, vaikka sillä näyttääkin olevan vaikutusta plasman resistiini-pitoisuuteen, mikä havaittiin toisessa osatyössä. Geenimuuntelulla oli havaittavissa yhteyttä aivovaltimosairauteen.

Kolmannessa osatyössä plasman resistiinipitoisuuden yhteyttä valtimonkovettumatautiin ja sen riskitekijöihin tutkittiin Pohjois-Pohjanmaalta kerätyssä aineistossa (n = 525). Plasman resistiinipitoisuudella ei havaittu itsenäistä yhteyttä kaulavaltimonseinämänpaksuuteen, joka kuvastaa alkuvaiheen valtimokovettumataudin tasoa. Tulehdukselliset merkit kuten veren valkosolujen määrä ja plasman CRP-pitoisuus liittyivät suurentuneeseen plasman resistiinipitoisuuteen mutta insuliiniresistenssimuuttujat eivät.

Neljännessä osatyössä tutkittiin resistiinin ilmentymistä. Resistiinin havaittiin ilmentyvän kaikissa keskeisissä valkosolutyypeissä ja lisäksi tulehdustekijät lisäsivät sen tuottoa. Erityisesti neutrofiileissä ja monosyyteissä resistiinin ilmentymistasot olivat korkeita. Endoteelisoluilla selvitettiin resistiinin vaikutuksia ateroskleroosiin liittyviin muutoksiin. Resistiini lisäsi monosyyttien kiinnittymistä endoteelisoluihin, mikä on tyypillinen ilmiö varhaiselle ateroskleroosille.

Tämän työn tulosten perusteella ihmisen resistiinillä ei ole merkittävää yhteyttä insuliiniresistenssiin ja tyypin 2 diabetekseen. Sen sijaan havainto resistiinin ilmentymisestä useammissa valkosolutyypeissä, kuin mitä aikaisemmin on raportoitu, ja yhteys tulehdustekijöihin osoittaa, että ihmisen resistiinin toiminta liittyy tulehdustiloihin. Resistiini aiheuttaa myös endoteelisoluissa samanlaisia ateroskleroosille altistavia muutoksia kuin muutkin tulehdustekijät. Tulevaisuudessa plasman resistiini-pitoisuutta voidaan mahdollisesti käyttää tulehdustilojen arvioinnissa.

Asiasanat: aikuistyypin diabetes, ateroskleroosi, geenitutkimus, resistiini, valkosolut

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Oulu, October 14th 2008 Anne Kunnari

Abbreviations

ADSF	adipocyte-specific secretory factor
ANCOVA	analysis of covariance
ANOVA	analysis of variance
BMI	body mass index
CABG	coronary artery bypass grafting
CAC	coronary artery calcification
CAD	coronary artery disease
CCA	common carotid artery
C/EBP	CAAT/enhancer binding protein
CI	confidence interval
CRP	C-reactive protein
CV	cardiovascular
CVD	cardiovascular disease
DNA	deoxyribonucleic acid
ET-1	endothelin 1
FACS	fluorescent-activated cell sorting
FIZZ	found in inflammatory zone
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GH	growth hormone
GHD	growth hormone deficient
GLUT	glucose transporter
$GHbA_{1c}$	glycosylated hemoglobin A_{1c}
HR	hazard ratio
hsCRP	highly sensitive CRP
HUVEC	human umbilical vein endothelial cell
ICA	internal carotid artery
ICAM-1	intercellular adhesion molecule 1
ICC	intra-class correlation coefficient
IGF-1	insulin-like growth factor 1
IGFBP-1	insulin-like growth factor binding protein 1
IL	interleukin
IMT	intima-media thickness
kb	kilo base pair
kDa	kilo dalton
LD	linkage disequilibrium

LDL	low density lipoprotein
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein 1
MI	myocardial infarction
MMP	matrix metalloprotease
mRNA	messenger ribonucleic acid
NE	primary human CD15 positive neutrophils and eosinophils
NK- κB	nuclear receptor-κB
NS	not significant
NSTEMI	non-ST-elevation myocardial infarction
OA	osteoarthritis
OPERA	Oulu Project Elucidating the Risk of Atherosclerosis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PECAM-1	platelet-endothelial cell adhesion molecule 1
PMNC	polymorphonuclear cells
RA	rheumatoid arthritis
RBC	red blood cells
RELM	resistin-like molecules
RETN	resistin gene
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT-PCR	real time polymerase chain reaction
SAP	stable angina pectoris
SD	standard deviation
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
STEMI	ST-elevation myocardial infarction
TLR	toll like receptor
TNFα	tumor necrosis factor α
TNFαR	tumor necrosis factor α receptor
T2D	type 2 diabetes
UAP	unstable angina pectoris
VCAM-1	vascular cell adhesion molecule 1
XCP	ten-cysteine protein

List of original articles

This thesis is based on the following original articles which are referred to in the text by their Roman numerals:

I Kunnari A, Ukkola O & Kesäniemi YA (2005) Resistin polymorphisms are associated with cerebrovascular disease in Finnish type 2 diabetic patients. Diabetic Medicine 22(5): 583–589.

II Ukkola O*, Kunnari A* & Kesäniemi YA (2008) Genetic variants at the resistin locus are associated with the plasma resistin concentration and cardiovascular risk factors. Regulatory Peptides 149(1–3): 56–59.

III Kunnari A, Ukkola O, Päivänsalo M & Kesäniemi YA (2006) High plasma resistin level is associated with enhanced hsCRP and leucocytes. Journal of Clinical Endocrinology and Metabolism 91(7): 2755–2760.

IV Kunnari A*, Savolainen E-R, Ukkola O, Kesäniemi YA & Jokela M*. The expression of human resistin in different leucocyte lineages is modulated by LPS and TNFα. Manuscript.

*Equal contribution

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1 Introduction

Consumption of too much energy rich food and lack of physical work and exercise are factors that most of us in the Western countries have become accustomed to during the last few decades. However, our bodies are not intented to this kind of "easy living" and health problems have followed in the form of not only of some gain in body weight but in severe overweight called obesity. It has been calculated that over 20% of adult Europeans are already overweight. With respect to the global population in 2007, 523 million people were estimated to be obese with body mass index (BMI) ≥ 30 kg/m^2 and this problem is increasing, unfortunately also among children. (Reviewed by James 2008.)

Obesity is a significant risk factor for other serious diseases such as diabetes, hypertension, atherosclerosis and arthritis (Ferrante 2007). The incidence of type 2 diabetes is increasing in parallel with obesity. Atherosclerosis is behind coronary artery disease (CAD) and cerebrovascular disease that are the leading causes of death in the world (Rader & Daugherty 2008). It is recognized that atherosclerosis, type 2 diabetes as well as obesity are common and multifactorial diseases and environmental, lifestyle, and genetic factors influence their development. Several genes are believed to contribute to the risk of suffering these multifactorial diseases. The human genome sequence project has provided new tools for the research of these common diseases. New genes have been found with the help of human deoxyribonucleic acid (DNA) sequence data and these are now being utilized in studies trying to understand the genetic basis of obesity related diseases.

Fat tissue, the amounts of which increase in obesity, was in the past considered to be simply a passive storage site for energy. However, new studies have provided novel insights into the functions of adipose tissue. (Reviewed in Ahima & Flier 2000). First adipocytes were discovered to secrete active peptides called adipokines such as leptin and adiponectin (reviewed in Meier & Gressner 2004). With the increasing efforts being made to understand the new functions of adipose tissue emerged a new peptide, resistin, this being first discovered to be produced by mouse adipocytes (Steppan *et al.* 2001a).

Resistin, is the focus of this thesis since animal studies indicated that resistin could induce insulin resistance and in this way, it could represent a link between obesity and type 2 diabetes (Steppan *et al.* 2001a). Since early studies emphasized the relatively clear differences between murine and human resistin, the major focus here was to clarify the role of resistin in its human context. During the

period that this study was carried out, the concept of resistin has changed fairly dramatically and this change in research focus can also be seen in the original studies. The following literature review focuses mainly on the literature related to human resistin.

2 Review of the literature

2.1 Discovery of resistin

A new gene family named resistin-like molecules (RELM) was discovered at almost the same time by different research groups. All of the published names of the gene family members are presented in Table 1. In this work, the official names will be used unless other aliases are appropriate.

Table 1. The nomenclature of resistin like molecules gene family members.

Official name	Official gene symbol	Other published aliases
Human		
resistin	*RETN*	XCP1, FIZZ3
resistin like beta	*RETNLB*	XCP2, FIZZ2
Mouse		
resistin	*Retn*	Xcp4, Fizz3, ADSF
resistin like alpha	*Retnla*	Xcp2, Fizz1
resistin like beta	*Retnlb*	Xcp3, Fizz2
resistin like gamma	*Retnlg*	Xcp1

Official nomenclature based on National Center for Biotechnology Information (NCBI) recommendations.

The work of Holcomb and co-workers (2000) was published first and they named the new genes as Found in Inflammatory Zone (FIZZ) 1–3 (Holcomb *et al.* 2000). They had discovered a new murine gene, Fizz1, while studying which gene expressions were increased during experimental asthma. After a nucleotide homology search, they identified two additional mouse genes (Fizz2 and Fizz3) and only two human genes (FIZZ2 and FIZZ3) though they stayed focused on Fizz1 known as resistin like alpha (Holcomb *et al.* 2000).

Shortly after the report of Holcomb *et al.*, Steppan and colleagues presented their findings (Steppan *et al.* 2001a). “The hormone resistin links obesity to diabetes” received more attention than Holcomb’s studies and resistin became the basis of the official names of the gene family. Steppan and colleagues discovered mouse resistin while looking for genes that were up-regulated during adipocyte differentiation and down-regulated by one class of anti-diabetic drugs, thiazolidinediones (Steppan *et al.* 2001a). In that study, mouse resistin was found to be increased in obesity and to impair the actions of insulin (Steppan *et al.* 2001a). These findings made resistin a hot topic of obesity and diabetes research. In addition, two other research groups, Kim (Kim *et al.* 2001) and Rajala (Rajala

et al. 2002) detected resistin when searching for genes induced during mouse adipocyte differentiation. Kim *et al.* named resistin as adipocyte-specific secretory factor, ADSF (Kim *et al.* 2001).

A third research line also led to the detection of resistin gene family. This time the research group of Koeffler (Chumakov *et al.* 2004, Kubota *et al.* 2000) found a still undiscovered fourth member of the gene family. This mouse gene was named as *Xcp1* (ten-cysteine protein) but it is better known as resistin like gamma *Retnlg* as described by others (Gerstmayer *et al.* 2003). *Retnlg* was identified when myelomonocytic genes that are regulated by CCAAT enhancer binding protein (C/EBP) ε were being screened. Based on the similar regulation and expression pattern, Chumakov and colleagues suggested that human resistin (*XCP1*) could be the functional homolog of mouse *Retnlg* (*Xcp1*) instead of mouse resistin (*Xcp4*) (Chumakov *et al.* 2004).

2.1.1 Gene and protein structure of resistin

RETN, a gene coding for human resistin, is located at chromosome position 19p13.3 (Yang *et al.* 2003). *RETN* is composed of four exons of which three participate in the formation of the protein (Fig. 1). The processed ribonucleic acid (RNA) product of *RETN* is 478 nucleotides in length (Ghosh *et al.* 2003). The protein translation starts from the exon II and ends in the middle of the exon IV leading to a product consisting of 108 amino acids. After the removal of the signal peptide with 16 amino acids, a protein composed of 92 amino acids is formed (Steppan *et al.* 2001a).

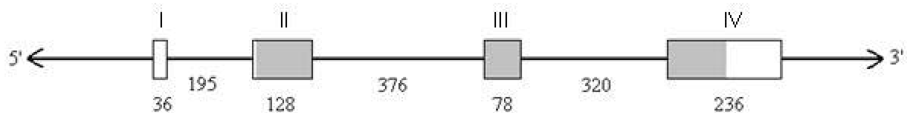

Fig. 1. Structure of human *RETN,* the gene coding for resistin. Exons (I–IV) are marked with squares and introns with the line. Amino acid coding regions are marked with gray. The length of exons and introns is stated under them in terms of nucleotides. Modified from Ghosh *et al.* (2003).

Common features to all members of the RELM gene family are an N-terminal signal peptide, a variable middle region, and a C-terminal cysteine-rich sequence. All of the family members have 10 cysteine residues in their cysteine-rich domain

which have identical spacing forming the following sequence: $CX_{11}CX_8CXCX_3CX_{10}CXCXCX_9CCX_{3\text{-}6}$, where C represents the cysteine and X is any other amino acid. (Holcomb *et al.* 2000.) All the other proteins except retnla (Holcomb *et al.* 2000) and the more recently discovered retnlg (Gerstmayer *et al.* 2003) have an additional cysteine residue (11th cysteine) in the middle domain, which has been reported to take part in dimerization of the protein (Banerjee & Lazar 2001).

There is 53% amino acid identity between human and mouse resistins (Holcomb *et al.* 2000) and 75.2% between human and pig (Dai *et al.* 2006). The two RELM members in human show only 47% amino acid identity (Holcomb *et al.* 2000). Naturally the conserved region of cysteine residues has more homology than other parts of the protein. For example, in the comparison of murine resistin and retnlb, the amino acid identities between signal sequences, middle regions, and cysteine-rich domains were 55, 18 and 54% (Steppan *et al.* 2001b).

Originally mouse resistin was calculated to form a 9.4 kDa protein (Holcomb *et al.* 2000). Shortly after the original discovery of resistin, Banerjee and Lazar (2001) reported that mouse resistin could form homodimers and that this dimerization required the presence of a disulfide bond between the 11th cysteine residues in the middle domains of the peptides. The other 10 cysteine residues were speculated to participate in the five intramolecular disulfide bonds that define the structure of the single resistin molecule. (Banerjee & Lazar 2001.) More detailed crystal structural studies have indicated that mouse resistin and retnlb are created from disulfide-rich β-sandwich "head" and α-helical "tail" segments (Patel *et al.* 2004). Resistin seems to form multimers but both dimer and trimer has been proposed to be the basic building component (Chen *et al.* 2002, Patel *et al.* 2004). Furthermore, there are conflicting results on whether oligomerization is required to generate physiologically active resistin (Graveleau *et al.* 2005, Juan *et al.* 2003, Patel *et al.* 2004).

Human resistin, estimated to be of 11.3 kDa size (Aruna *et al.* 2003), has been claimed to hold the same structural properties as mouse resistin. The corresponding 11th cysteine has been reported to mediate the dimerization and the human protein is also proposed to be a mixture of α-helical and β-sheet conformations (Raghu *et al.* 2004). Human resistin has also been detected to form oligomers (Aruna *et al.* 2003, Raghu *et al.* 2004). Gerber and co-workers described five different molecular weight forms of resistin in human plasma, from which the large 660 and 55 kDa forms showed the highest peaks in the size exclusion chromatography (Gerber *et al.* 2005). Molecule of 660 kDa appears

extremely large considering the size of the resistin monomere (11.3 kDa). Possibly laboratory methods have caused aggregation, since other studies have not reported such a large multimere. It is not known whether these different molecular forms of human resistin express differences in biological activity.

2.1.2 Expression pattern of resistin

Resistin was initially discovered in mouse. Expression studies detected its presence in mouse adipose tissue samples (Holcomb *et al.* 2000, Kim *et al.* 2001, Rajala *et al.* 2002, Steppan *et al.* 2001a) as well as in the mouse 3T3-L1 adipocyte cell line (Kim *et al.* 2001). Moreover, mouse resistin was reported to be up-regulated during adipocyte differentiation (Haugen *et al.* 2001, Kim *et al.* 2001, Steppan *et al.* 2001a). Both murine and human resistin are readily detectable in blood (Steppan *et al.* 2001a) and these two proteins were first believed to possess the same properties.

Resistin was clearly present in mouse adipose tissue but the samples of human adipose tissue produced controversial results, some research groups detecting clear expression (Kusminski *et al.* 2007, McTernan *et al.* 2002a, McTernan *et al.* 2002b, McTernan *et al.* 2003, Pagano *et al.* 2005) and others only borderline or no expression at all (Fain *et al.* 2003, Nagaev & Smith 2001, Ort *et al.* 2005, Savage *et al.* 2001). Furthermore, some of these studies have detected expression of resistin in adipose tissue samples but not in adipocytes (Fain *et al.* 2003, Savage *et al.* 2001) suggesting that it is some other cell type in the tissue that is producing resistin. Indeed, shortly after the discovery of resistin, it was reported to be clearly present in peripheral blood mononuclear cells (PBMCs) (Kaser *et al.* 2003, Lehrke *et al.* 2004, Nagaev & Smith 2001, Patel *et al.* 2003, Savage *et al.* 2001, Yang *et al.* 2003) and this observation has been confirmed in more recent studies (Nagaev *et al.* 2006, Xu *et al.* 2007). Porcine resistin, which is more homologous to human resistin than murine resistin, has also been shown to be expressed strongly in leucocytes (Dai *et al.* 2006).

Positive signals of resistin in adipose tissues have been claimed to be attributable to tissue macrophages and/or other blood leucocytes. Curat and colleagues showed that after immunoselection, the detected expression of resistin in the adipose tissue samples was predominantly produced by monocyte/macrophages instead of adipocytes (Curat *et al.* 2006). However, some recently published studies reporting expression of resistin in separated primary adipocytes did not rule out this possibility (e.g. Pagano *et al.* 2005). In addition,

some reports have failed to discuss the possible effect of leucocyte contamination and have presented their results as if they were obtained from pure adipocytes even though they detected not only expression of resistin but also the leucocyte marker CD45 in their human adipose tissue samples (Kusminski *et al.* 2007, McTernan *et al.* 2003).

In addition to adipose tissue expression, human resistin has been detected in several other tissues, such as adrenal gland, bone marrow, breast, brain, colon heart, kidney, liver, lung, pancreas, placenta, prostate, skeletal muscle, small intestine, spleen, stomach, testis, thymus, thyroid gland and uterus (Chumakov *et al.* 2004, Nohira *et al.* 2004, Patel *et al.* 2003). Some studies have reported that human resistin is not expressed by skeletal muscle, hepatic, or endothelial cells (Bertolani *et al.* 2006, Nagaev & Smith 2001, Savage *et al.* 2001). Positive signals of resistin in several tissues are probably attributable to the tissue leucocytes, as was demonstrated to be the case in adipose tissue samples.

Plasma resistin concentration and its normal variations

The normal plasma resistin concentration range in healthy humans is still unclear. Reported mean values have varied between 2–40 ng/ml (e.g. Burnett *et al.* 2006, Fehmann & Heyn 2002, On *et al.* 2007, Silha *et al.* 2003, Stejskal *et al.* 2003). This large range is probably caused by different combinations of antibodies, standards etc. used in the immunoassays. Most studies have presented average concentrations between 5–10 ng/ml (e.g. Anderson *et al.* 2007, Baker *et al.* 2006, Lee *et al.* 2007, Yaturu *et al.* 2006). Variations in plasma resistin levels have been studied and its level appears to be fairly stable throughout the day (Reilly *et al.* 2005). This is partly because resistin does not seem to be regulated by eating since 48 hour fasting did not affect plasma resistin levels nor was it altered by leptin administration (Lee *et al.* 2003). In addition, the level of resistin did not correlate with total energy or macronutrient intake (Yannakoulia *et al.* 2003). Furthermore, olive oil consumption study showed that the contents of phenolic compounds in different olive oils were unable to change the plasma resistin level (Machowetz *et al.* in press).

The variation over a longer time period has been investigated by Lee and co-workers (Lee *et al.* 2007). They have reported that there were seasonal changes in the plasma resistin level. The variability in plasma resistin level over a year seems to be higher than that occurring in a single day. Four measurements obtained at different time points in the year did not differ significantly but the intra-class

correlation coefficient (ICC) value of 0.49 was one of the lowest of all the measured variables. In comparison, the ICC values were 0.81, 0.59, and 0.48 for adiponectin, highly sensitive C-reactive protein (hsCRP), and TNFα, respectively. (Lee *et al.* 2007.) The effect of the common cold on plasma resistin level has not been studied but since resistin is produced by inflammatory cells it could be speculated that this could have an effect on the resistin level and thereby introduce variability into the seasonal resistin concentration.

Some studies have shown gender-related differences in the plasma resistin levels (Lee *et al.* 2003, Silha *et al.* 2003, Yannakoulia *et al.* 2003). Women have been claimed to have higher plasma resistin levels and therefore this has been suggested to be attributable to female sex hormones. The plasma resistin concentration has been reported to increase during pubertal maturation and to correlate with changes in testosterone and estrogen in children and adolescents (Gerber *et al.* 2005). It has also been shown that plasma resistin levels are highest in adult women compared to men or boys and girls still in puberty (Martos-Moreno *et al.* 2006). Menopausal status has been stated to influence the plasma resistin level (Chu *et al.* 2006). However, neither estrogen nor testosterone replacement therapy has been shown to have any effect on the plasma resistin level (Kapoor *et al.* 2007, Kunnari *et al.* 2008).

The effect of other hormones e.g. growth hormone (GH) treatment on plasma resistin levels has been studied. Short term (1 month) therapy has been shown to increase the plasma resistin concentration in GH-deficient (GHD) children (Nozue *et al.* 2007) whereas long term (1–2 years) treatment does not seem to influence resistin levels in GHD adults (Hana *et al.* 2004) or in short children born small-for-gestational-age (Willemsen *et al.* 2007).

2.2 Genetic association studies

Several of the common diseases which have a significant impact on public health such as type 2 diabetes are considered to be complex diseases, meaning that they are the result of a complex set of susceptibility alleles of several genes, environmental factors and their interactions. Promising results from genetic approaches have been obtained in monogenic diseases where mutation/mutations in one gene are sufficient to predispose to the disease. In complex diseases, it is believed that several common alleles with smaller individual effects may influence the risk of the disease. The small contribution of a single gene and the possible high frequency of the risk allele at the population level, make it difficult

to identify the predisposing loci of complex and common diseases. (E.g. Lander & Schork 1994, Risch 2000.)

Most of the success in monogenic diseases has been obtained from linkage mapping studies that are based on pedigree data where the transmission and co-segregation of disease and markers distributed genome widely are evaluated. Those known markers that are close to the disease locus should be transmitted together in the pedigree. Linkage mapping has not worked efficiently in studies of complex diseases since the power of the test is not sufficient to detect loci having only a small effect on the trait. (Schork *et al.* 2000.) Another approach requiring denser map of markers is based on linkage disequilibrium (LD) and it does not require families. In the LD mapping, the association of the marker and the disease loci close to each other can be detected if the recombinations during generations have not disintegrated the linkage. (Gray *et al.* 2000.) In relatively newly-established populations, the LD is reported to extend wider than is the case in older populations. The late settlements of Finland have shown LD on average of 60–70 kb whereas in African populations it has been reported to be less than 10 kb. (Reviewed by Varilo & Peltonen 2004.)

In the linkage and LD mapping approaches, it is assumed that the markers that are used are indirectly associated with the trait. Alleles of a polymorphic site can also be relevantly associated with the trait by having a functional effect on the gene function. (Schork *et al.* 2000.) Sometimes the information from the gene function can create the hypothesis on the possible link to a disease. In these candidate gene studies, the association to a disease is commonly tested with the case-control arrangement, where the allele or genotype frequencies of the candidate variation/s in the gene area are compared between the groups. (Hegele 2002.)

Single nucleotide polymorphisms (SNPs) are one type of variation present in the genome. SNPs are produced by single nucleotide mutations. A mutation is defined as an SNP when the variant occurs with a population frequency greater than 1%. SNPs can be up to tetra-allelic but usually they are only bi-allelic. (Brookes 1999.) It has been estimated that SNPs are distributed along the genome at a distance of 0.3–1 kb, making SNPs the most frequent variations in the genome (Schork *et al.* 2000). However, there is considerable variability in the SNP frequency between genes, which is produced by different selection pressures on each gene. In addition, the bias from more studies on coding regions must be taken into account when estimations are made from the SNP frequency. (Gray *et*

al. 2000.) Since SNPs are common and straight forward to genotype, they have become intensively used in genetic studies.

2.2.1 Single nucleotide polymorphisms (SNPs) in RETN

The importance of the genetic component in the expression of resistin and its concentration in blood has not been widely studied. The genetic background seems to explain part of the variation in plasma resistin levels. Menzaghi and co-workers reported that the plasma resistin concentration is highly heritable, with 70% of its variability explained by genetic factors (Menzaghi *et al.* 2006). A similar result was obtained by Pantsulaia and colleagues who stated that the additive genetic effect was highly significant for resistin and that 66% of the phenotypic variance was explained by genetic factors (Pantsulaia *et al.* 2007).

Most of the genetic studies of resistin are based on SNPs. Several SNPs in *RETN* have been described in the literature, with most of them being in non-coding regions of the gene such as in the 5' promoter region (Engert *et al.* 2002, Ma *et al.* 2002, Osawa *et al.* 2002, Pistilli *et al.* 2007, Pizzuti *et al.* 2002, Wang *et al.* 2002, Xu *et al.* 2007), intron 1 (Xu *et al.* 2007), intron 2 (Cao & Hegele 2001, Engert *et al.* 2002, Ma *et al.* 2002, Osawa *et al.* 2002, Pistilli *et al.* 2007, Pizzuti *et al.* 2002, Wang *et al.* 2002, Xu *et al.* 2007), intron 3 (Engert *et al.* 2002, Ma *et al.* 2002, Pizzuti *et al.* 2002, Wang *et al.* 2002, Xu *et al.* 2007), and in the 3' untranslated region (UTR) (Cao & Hegele 2001, Engert *et al.* 2002, Sentinelli *et al.* 2002, Wang *et al.* 2002, Xu *et al.* 2007). In addition, one GAT or ATG repeat polymorphism has been detected (Ma *et al.* 2002, Pizzuti *et al.* 2002, Wang *et al.* 2002, Xu *et al.* 2007). In these screenings for polymorphisms, *RETN* has been sequenced in a total of over 500 healthy and diabetic patients of Caucasian and Asian origins. Only one of the discovered variants actually leads to an amino acid composition (Pizzutti 2002). The frequency of this missense variant was recorded by Pizzutti and co-workers to be below 2% meaning that it was too rare for efficient association study (Pizzuti *et al.* 2002). In addition, two synonymous SNPs in intron 2 have been found from the NCBI dbSNP database. The lack of amino acid changing polymorphisms suggests that the resistin protein structure is very sensitive to amino acid changes.

2.2.2 RETN promoter variant SNP-420C>G

With respect to the reported resistin variants, the mostly extensively studied has been the promoter variant SNP-420C>G. This promoter variant is located 420 nucleotides upwards from the translation starting point in exon 2. The promoter with the rarer G allele in SNP-420C>G has been reported to be more active in several studies (Azuma *et al.* 2004, Cho *et al.* 2004, Chung *et al.* 2005, Osawa *et al.* 2004, Smith *et al.* 2003). Studies utilizing algorithms have proposed that this leads to a change in the binding sites of several transcription factors (Engert *et al.* 2002, Smith *et al.* 2003). Functional binding studies have been done with stimulatory proteins (Sp)-1 and 3, which bind to the promoter. Their binding has been described to be influenced by SNP-420C>G. Sp-1 and 3 were discovered to bind efficiently only to the G-allele sequence and after binding to increase the activity of the promoter. (Chung *et al.* 2005, Osawa *et al.* 2004).

It seems likely that the more active promoter with the SNP-420C>G G allele is the reason for several observations of higher plasma resistin concentration in the G allele carriers (Axelsson *et al.* 2006, Azuma *et al.* 2004, Cho *et al.* 2004, Osawa *et al.* 2004, Osawa *et al.* 2005, Osawa *et al.* 2007, Qasim *et al.* in press, Yamauchi *et al.* 2008). However, in contrast to these studies, it has also been reported that the genotypes of SNP-420C>G do not influence the plasma resistin concentration in Italian subjects (Menzaghi *et al.* 2006, Norata *et al.* 2007a). Furthermore in a small study of polycystic ovary syndrome patients no association was detected between SNP-420C>G genotype and the serum level of resistin (Escobar-Morreale *et al.* 2006).

A similar discrepancy exists in the results of SNP-420C>G and resistin messenger RNA (mRNA) expression in mononuclear cells. In healthy subjects, relative resistin mRNA expression was detected to be higher in monocytes of those subjects with the G/G genotype and this was positively correlated with the plasma concentration (Osawa *et al.* 2005), whereas in patients with renal dysfunction no effect of SNP-420 C>G was detected (Norata *et al.* 2007a). Both of these studies contained a relatively small number of subjects and thus the effect of this SNP on resistin expression in monocytes needs to be further studied in larger study groups. In abdominal subcutaneous fat samples, the G/G homozygotes have also been reported to possess higher mRNA expression levels (Smith *et al.* 2003). Since the prevailing view is that human adipocytes do not express resistin (Nagaev *et al.* 2006) the detected expression is most likely to be targeting to adipose tissue macrophages.

2.2.3 *Genetic association studies – contradictory results about the association of resistin to type 2 diabetes*

Since mouse resistin seemed to be related to insulin resistance, several genetic studies on human *RETN* have focussed on type 2 diabetes. These studies have not provided strong support for the hypothesis that resistin would be an important factor in type 2 diabetes. In Table 2, the largest studies on the SNP-420C>G and its connection to diabetes have been summarised. In most of these case-control studies, neither allele nor genotype frequencies in the SNP-420C>G differed between nondiabetic subjects and subjects with type 2 diabetes.

Table 2. Results from *RETN* SNP-420C>G association studies. Large (over 100 subjects) type 2 diabetes related studies have been included.

Author	Study population	Difference in allele / genotype frequencies	G allele linked with high glucose / insulin	G allele associated with BMI
Cho *et al.* 2004	411 Korean cases with type 2 diabetes and 173 nondiabetic controls.	No	No / No	No
Conneely *et al.* 2004	781 Finnish type 2 diabetes cases and 409 controls.	No	–	–
Engert *et al.* 2002	179 vs.180 Caucasian and 452 vs. 433 Scandinavians type 2 diabetic cases and nondiabetic controls, respectively.	No / No	–	No, No
Ma *et al.* 2002	312 Caucasian type 2 diabetes cases and 303 controls.	No	–	No
Ochi *et al.* 2007	2610 Japanese type 2 diabetes cases and 2502 controls.	Yes [1]	–	–
Osawa *et al.* 2004	397 Japanese cases with type 2 diabetes and 406 controls.	Yes [2]	–	–
Xu *et al.* 2007	Cross-sectional study: 312 Chinese subjects with normal glucose tolerance (NGT) and 312 with impaired glucose tolerance (IGT).	No	No / No [4]	No
	Prospective study (5 years): 539 subjects of the cross-sectional study from which 280 were NGT and 259 IGT at that time.	Yes [3]	–	–

[1] Differences were only significant in subjects < 40 years., [2] G/G genotype was associated with type 2 diabetes., [3] The G allele was associated with progression of glycaemia., [4] G allele was associated with 2 h glucose and insulin in oral glucose tolerance test.

Other *RETN* polymorphisms have not been studied as widely. Two studies comparing genotype frequencies of the 3' untranslated region SNP+62G>A between subjects with type 2 diabetes and nondiabetic controls depicted contradictory results (Gouni-Berthold *et al.* 2005, Tan *et al.* 2003). Tan and co-workers reported a lower frequency of the A allele in the subjects with type 2 diabetes in Chinese population (Tan *et al.* 2003) whereas in Europeans no such difference was observed (Gouni-Berthold *et al.* 2005). In addition, no difference was reported between subjects with normal or impaired glucose tolerance in a Chinese population (Xu *et al.* 2007). Furthermore, studies on all the other SNPs in *RETN* have not detected any association with type 2 diabetes (Cho *et al.* 2004, Conneely *et al.* 2004, Engert *et al.* 2002, Ma *et al.* 2002, Ochi *et al.* 2007, Osawa *et al.* 2002, Sentinelli *et al.* 2002, Wang *et al.* 2002).

The SNP-420C>G has also been studied in relation to some diseases other than type 2 diabetes. In the case of obesity, no difference in allele frequencies between normal weight (BMI < 25) (n = 251) and overweight (BMI ≥ 25) (n = 334) subjects was observed in Caucasian men but in women the G allele was reported to be more frequent in the normal weight group (Mattevi *et al.* 2004). In a large population based cohort of 1542 individuals, obesity and metabolic syndrome were detected to be more prevalent in G/G homozygotes of the SNP-420C>G (Norata *et al.* 2007a).

In the more recent studies, the association of resistin to coronary heart disease and inflammation has also been studied. Tang and colleagues studied 225 Chinese coronary heart disease cases and controls. They discovered that the allele and genotype frequencies differed between groups and that the G-allele carriers had increased risk for coronary heart disease compared to C-allele homozygotes (Tang *et al.* 2008). A conflicting result was obtained from a study of patients with myocardial infarction (MI) and controls where no such difference between groups was observed (Norata *et al.* 2007a). Furthermore, in healthy Caucasian subjects with a family history of premature CAD (n = 851) SNP-420C>G was not associated with coronary artery calcification (CAC) (Qasim *et al.* in press). However, in the same study, GGT and AGT haplotypes of SNP-852A>G, -420C>G and +157C>T were associated with soluble TNFα receptor 2 and CRP, respectively, when compared to the most common haplotype ACC (Qasim *et al.* in press).

2.3 Resistin and inflammation

Our body is constantly under attack by different pathogens. Our immune defence is based on innate and adaptive immune systems that work through different white blood cells that all originate from the bone marrow hematopoietic stem cells (Fig. 2). Innate immunity represents the first line defence and this is provided by macrophages, the mature form of monocytes, and granulocytes (polymorphonuclear leucocytes), with neutrophils being the most prominent of cells. These phagocytic cells remove and destroy pathogens and initiate the inflammatory process through the cytokines and chemokines they produce. Dendritic cells that reside inside the tissues ingest also pathogens in the location of infection and after activation they move to lymph nodes and there present pathogen antigens to T lymphocytes simultaneously activating these cells. Natural killer cells proliferating from lymphoid progenitors are also part of the innate immunity system. They can recognise and remove abnormal cells. (Reviewed in Janeway *et al.* 2005.)

The functions of the innate immunity system are crucial during the early control of infection since the adaptive immune system depending on lymphocytes is effective after about 5 days. On the other hand, adaptive immunity with B and T lymphocytes is required for pathogen specific and lifelong immunity. Naive T lymphocytes that each possesses a specific antigen receptor are recirculating through the lymphoid organs. When an antigen is presented to the T cell by dendritic cells, macrophages or B lymphocytes, it proliferates and differentiates into an antigen specific effector cell. Cytotoxic T killer cells and T helper cells are formed from effector T cells. T helper cells activate macrophages, making them more efficient and they activate B cells to differentiate into antibody producing plasma cells or memory B cells. The antibodies produced by B cells are a key feature of the defence system when a pathogen is encountered for the second time. Lymphocytes possess specific antigen receptors and those cells that have receptors against self-antigens need to be eliminated during cell development. However, some autoreactive lymphocytes are not eliminated and they can become activated in autoimmunity diseases. (Reviewed in Janeway *et al.* 2005.)

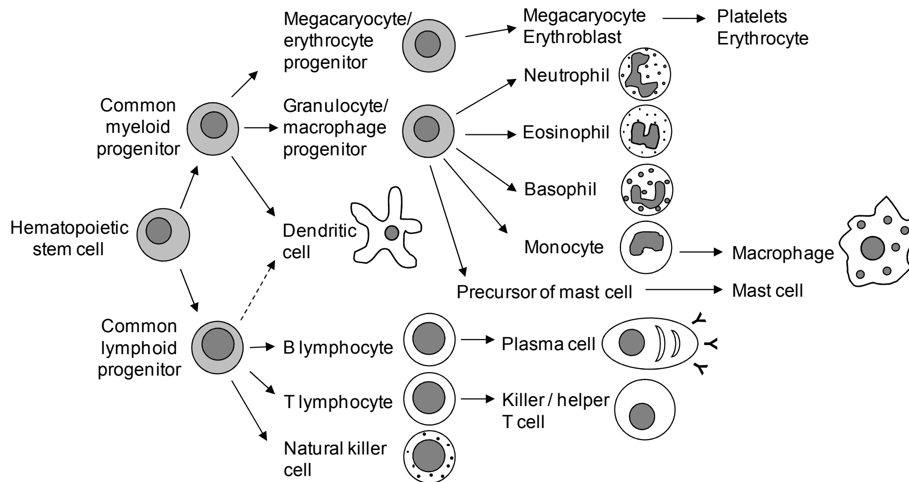

Fig. 2. Family tree of major white blood cell populations. Cells coloured in gray reside in bone marrow whereas the non-shaded cell types followed are present in blood, lymph nodes, or in tissues. Modified from Janeway *et al.* 2005.

Several studies have convincingly shown that human resistin is produced by PBMCs instead of adipocytes (e.g. Nagaev *et al.* 2006). PBMCs can be separated by density gradient centrifugation and this fraction is largely composed of monocytes and lymphocytes. Therefore an increasing effort has been exerted in evaluating levels of resistin in different inflammatory diseases. In this chapter, *in vitro* and population based studies on the connection of resistin to inflammation are reviewed. Atherosclerosis and obesity that can also be considered low grade inflammatory diseases are discussed in their own chapters.

2.3.1 Resistin is induced by inflammatory factors in vitro

Modulators of resistin expression have been tested in several cell culture studies. Mostly primary PBMC or monocytic cancer cell lines U937 and THP-1 have been used in these experiments. Several factors that are considered to be proinflammatory or inflammation-related have been reported to influence the expression of resistin. The effects of bacterial surface lipopolysaccharides (LPS) in resistin expression have been studied in primary PBMCs (Bokarewa *et al.* 2005, Kaser *et al.* 2003, Lu *et al.* 2002, Sundén-Cullberg *et al.* 2007) and PBMC originated macrophages (Lehrke *et al.* 2004) and all the studies have detected an increase in the expression of resistin. However, in monocytic U937 cells that were

differentiated into macrophages, Yang and co-worker did not detect any increase in resistin mRNA after LPS treatment (Yang *et al.* 2003). The proinflammatory cytokine, TNFα, has also been shown to induce the expression of resistin in PBMCs (Bokarewa *et al.* 2005, Kaser *et al.* 2003, Lehrke *et al.* 2004).

The effects of other cytokines on resistin levels are inconsistent. In PBMCs, the expression of resistin has been reported either not to change (Bokarewa *et al.* 2005) or to be up-regulated by interleukin (IL) -6 and IL-1β treatments (Kaser *et al.* 2003). The difference between these results might be a consequence of the lower doses of cytokines used by Bokarewa and colleagues (Bokarewa *et al.* 2005). In the monocytic THP-1 cell line, LPS and TNFα did not affect the mRNA expression of resistin (Bokarewa *et al.* 2005) due to the fact as shown by others that these cells do not express resistin (Nagaev *et al.* 2006, Rae & Graham 2006). Furthermore, C-reactive protein (CRP) has been shown to stimulate mRNA and protein expressions of resistin in a dose- and time-dependent manner in PBMCs (Hu *et al.* 2007a).

In human adipose tissue derived adipocytes, insulin has been reported to increase secretion but not mRNA expression of resistin and this increase was shown to be attenuated by the drug rosiglitazone (McTernan *et al.* 2003). In addition, LPS has been shown to stimulate the secretion of resistin from adipocytes (Kusminski *et al.* 2007). In both of these studies, the authors detected also mRNA expression of leucocyte marker CD45 in the cultured cells meaning that insulin could have increased the secretion of resistin in tissue macrophages, which had been co-isolated together with adipocytes.

2.3.2 Resistin stimulates inflammation related changes in cells

Inflammatory cytokines stimulate human resistin as described above but resistin itself promotes expressions of these cytokines as well (Fig. 3). In human PBMCs and THP-1 cells, resistin induced the mRNA expressions and secretions of TNFα, IL-6, IL-1β, and itself (Bokarewa *et al.* 2005) whereas in U937 cells, resistin has been shown to up-regulate secretion of TNFα and IL-12 (Silswal *et al.* 2005). Therefore resistin seems to possess auto/paracrine properties since both human PBMCs and U937 monocytes that produce resistin also respond to resistin.

Resistin has also been reported to increase the production of TNFα, IL-6, and Toll like receptor (TLR) 2 protein but not TLR 4 in adipocytes (Kusminski *et al.* 2007). Nagaev and co-workers (Nagaev *et al.* 2006) showed that in addition to TNFα and IL-6, adipose tissue exposed to resistin expressed more IL-8, monocyte

chemoattractant protein (MCP) 1, matrix metalloprotease (MMP) 3, and pre-B-cell colony enhancing factor. Proinflammatory changes have also been reported in hepatic stellate cells in response to resistin. Bertolani and co-workers showed that secretion of MCP-1 and IL-8 increased after resistin treatment (Bertolani *et al.* 2006). However, they did not detect any increase in cell proliferation (Bertolani *et al.* 2006).

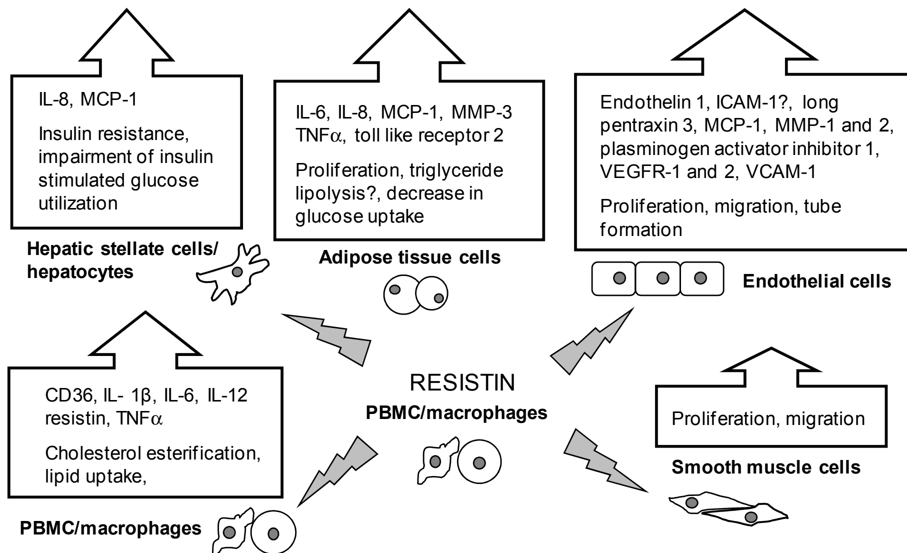

Fig. 3. Resistin promotes changes in different human cells. ICAM-1, intercellular adhesion molecule 1; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; MMP, matrix metalloprotease; PBMC, peripheral blood mononuclear cell; TNFα, tumor necrosis factor alpha; VCAM-1, vascular cell adhesion molecule 1; VEGFR, vascular endothelial growth factor receptors; ?, contradictory results.

Resistin affects several different cells and this is summarized in Fig. 3. How the effects of resistin are mediated into these cells can only be speculated since the receptor for resistin is still undiscovered. Regardless of the lack of a receptor, it has been shown in several studies that resistin can activate transcription factor nuclear receptor-κB (NK-κB). In monocytic U937 cells (Haugen & Drevon 2007, Silswal *et al.* 2005), PBMCs (Bokarewa *et al.* 2005), adipocytes (Kusminski *et al.* 2007) as well as in bovine aortic endothelial cells, resistin has been shown to increase the activation or expression of NK-κB. The induction of IL-6 by resistin was abrogated by the NK-κB inhibitor in PBMCs (Bokarewa *et al.* 2005) as was

the TNFα secretion from U937 cells (Silswal *et al.* 2005). NK-κB signaling pathway has been reported to be involved also in the induction of MCP-1 and IL-8 by resistin in hepatic stellate cells (Bertolani *et al.* 2006). NK-κB pathways are known to be activated by pro-inflammatory cytokines (Cilloni *et al.* 2007). The reported effects of resistin on NK-κB are support for a role of resistin as one of these cytokines.

2.3.3 Increased levels of resistin are associated with acute and chronic inflammatory diseases

In *in vitro* experiments (Bokarewa *et al.* 2005, Kaser *et al.* 2003, Lehrke *et al.* 2004, Lu *et al.* 2002, Sundén-Cullberg *et al.* 2007), resistin was discovered to be up-regulated by bacterial LPS. Two small studies by Lehrke (2004) and Anderson (2007) with colleagues have extended this hypothesis to healthy humans. In support of the *in vitro* observations, endotoxin infusion was found to increase the resistin concentration in plasma as well as the mRNA level in PBMCs (Anderson *et al.* 2007, Lehrke *et al.* 2004). In both of these experiments, the plasma resistin concentration peaked 6–12 hours after the LPS infusion. In more severe cases of endotoxemia such as sepsis and septic shock, the resistin concentration has been reported to remain elevated for up to two weeks after admission to intensive care (Sundén-Cullberg *et al.* 2007).

In addition to experimentally produced endotoxemia, an increased plasma concentration of resistin has been detected in acute inflammatory diseases. It has been reported that the plasma resistin level is higher in group of patients with respiratory tract inflammation such as bronchitis and pneumonia compared to group of controls or subjects with type 2 diabetes (Stejskal *et al.* 2003). The resistin concentration is not only correlated with the presence of inflammation but it seems to correlate with the severity of the inflammation. This kind of correlation was reported in severe sepsis and septic shock patients where resistin correlated with the disease severity scores (Sundén-Cullberg *et al.* 2007). In these severe inflammatory cases, resistin concentrations were over 20 times higher than in healthy controls (Sundén-Cullberg *et al.* 2007).

Chronic inflammation is one aspect of several diseases affecting different parts of the body. In some of these human diseases such as rheumatoid arthritis (RA) (Bokarewa *et al.* 2005, Migita *et al.* 2006, Senolt *et al.* 2007), inflammatory bowel disease (Konrad *et al.* 2007), and asthma (Larochelle *et al.* 2007) resistin has also been studied. RA is a chronic inflammatory disease of joints. Schäffler

and colleagues (2003) were the first to report that resistin is measurable from synovial fluid and is actually increased in RA patients in comparison with patients with osteoarthritis (OA) (Schäffler *et al.* 2003). A similar difference between RA and OS or healthy controls in the resistin concentration in synovial fluid and also in plasma has been reported by others (Bokarewa *et al.* 2005, Migita *et al.* 2006, Senolt *et al.* 2007). Only Otero and co-workers have not found any significant difference in the plasma resistin concentration between RA patients and healthy controls (Otero *et al.* 2006).

In all the studies where both synovial fluid and plasma resistin concentrations have been measured, an interesting difference has been discovered. In RA patients, synovial fluid was found to contain a significantly higher amount of resistin than plasma whereas in non-inflammatory OA patients the situations was the opposite (Bokarewa *et al.* 2005, Presle *et al.* 2006, Senolt *et al.* 2007). This observation suggests that resistin concentration can be higher locally in the actual site of inflammation than in the systemic blood circulation. The synovial fluid resistin concentration has been reported to be associated with the IL-6 level and synovial leucocyte count (Bokarewa *et al.* 2005) as well as synovial fluid CRP concentration (Schäffler *et al.* 2003).

In chronic inflammatory diseases other than arthritis, resistin has been less frequently studied but the results reveal a similar connection between increased resistin and inflammation. In inflammatory bowel disease cases (Crohn's disease and ulcerative colitis), the plasma resistin concentration was noted to be higher than in healthy controls (Karmiris *et al.* 2006, Konrad *et al.* 2007). In addition, resistin concentrations were decreased along with the remission of the disease after infliximab treatment which blocks the actions of TNFα (Karmiris *et al.* 2007). Individual studies have also shown higher plasma resistin concentrations compared to healthy controls in patients with asthma (Larochelle *et al.* 2007) and periodontitis (Saito *et al.* 2008).

The level of resistin is increased in several inflammatory diseases as described above. Furthermore, the plasma resistin concentration has been linked to many factors known to be involved in inflammation which supports the hypothesis that resistin is also an inflammatory cytokine. In different study designs and study groups, the plasma resistin level has been reported to be positively correlated with plasma CRP (Al-Daghri *et al.* 2005, Aquilante *et al.* 2008, Efstathiou *et al.* 2007, Hu *et al.* 2007b, Konrad *et al.* 2007, Lubos *et al.* 2007, Malyszko *et al.* 2007, Ohmori *et al.* 2005, Piestrzeniewicz *et al.* 2008, Pilz *et al.* 2007, Pischon *et al.* 2005, Reilly *et al.* 2005, Shetty *et al.* 2004, Stejskal *et*

al. 2003, Yaturu *et al.* 2006), TNFα (Koçak *et al.* 2007, Lee *et al.* 2007, Migita *et al.* 2006, Stejskal *et al.* 2003, Sundén-Cullberg *et al.* 2007, Yaturu *et al.* 2006), soluble TNFα receptor 1 (Vendrell *et al.* 2004) and 2 (Reilly *et al.* 2005, Vendrell *et al.* 2004), IL-6 (Harsch *et al.* 2004, Hui-Bing *et al.* 2006, Lee *et al.* 2007, Malyszko *et al.* 2007, Reilly *et al.* 2005, Stejskal *et al.* 2003, Sundén-Cullberg *et al.* 2007), IL-8 (Lee *et al.* 2007, Sundén-Cullberg *et al.* 2007), IL-10 (Sundén-Cullberg *et al.* 2007), and serum amyloid A (Hui-Bing *et al.* 2006).

2.4 Resistin in atherosclerosis

Atherosclerosis is considered to be a complex and chronic inflammatory disease. It is a disease of large and medium-sized, elastic and muscular arteries (reviewed by Ross 1999). Atherosclerosis is a progressive disease where the accumulation of lipids and fibrous tissue forms lesions on the arterial wall, which can lead to the narrowing of the arteries (Fig. 4) (reviewed by Libby 2002). Atherosclerosis can develop for decades but the acute complications are the results of the rupture of the lesion and the subsequent thrombosis which can be clinically manifested as MI or stroke (reviewed in Glass & Witztum 2001). The concepts of atherosclerosis have changed in the last decades. Initially, atherosclerosis was considered predominantly as a lipid disease due to the clear link between hypercholesterolemia and atheroma but the increasing knowledge from cell level changes moved the emphasis to growth factors and smooth muscle cell (SMC) proliferation. The latest change in atherosclerosis research field has been the introduction of inflammation as a major factor in the disease. (Reviewed by Libby 2002.)

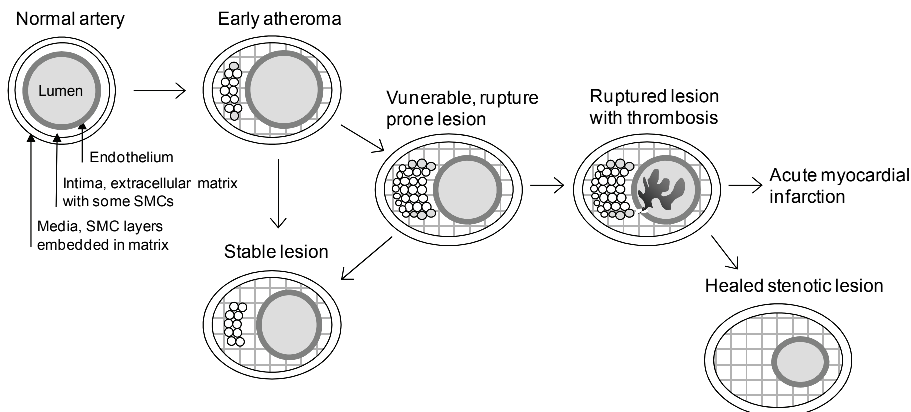

Fig. 4. The progression of arterial lesion from a normal artery to a fibrous lesion with a lipid core. Modified from Peter Libby (2002).

According to Ross, endothelial dysfunction is the first step of atherosclerosis (response-to-injury hypothesis) (Ross 1999). Endothelial dysfunction can be created by elevated and modified low density lipoprotein (LDL), free radicals, hypertension, diabetes, infections, and genetic alterations. Endothelial dysfunction changes the normal functions of endothelium by increasing its permeability, secretion of cytokines, and growth factors as well as decreasing anticoagulant properties. These modifications in the endothelium induce leucocyte accumulation into the areas of endothelial dysfunction. The earliest form of atherosclerosis, the fatty streak that can be found already in children is formed from macrophages, foam cells and T lymphocytes accompanied by extracellular lipids in persons with hypercholesterolemia. (Reviewed by Ross 1999.)

The accumulation of leucocytes is a key feature of atherosclerosis (Fig. 5). Studies have shown that MCP-1 secreted by activated endothelium is one of the key chemokines recruiting leucocytes through the CCR2 receptor on their surface (reviewed by Charo & Taubman 2004). In addition, IL-8 and oxidized LDL have been reported to attract leucocytes (reviewed in Glass & Witztum 2001). The recruitment of leucocytes inside the intima consists of several steps involving different adhesion molecules. First endothelial selectins (P and E-selectin) and their ligands in leucocytes are responsible for the initial loose connection and rolling of the leucocytes on the endothelium allowing them to make contanct with endothelial chemokines. These chemokines activate leucocyte integrins that

become firmly attached to the intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule (VCAM) 1, and the platelet-endothelial adhesion molecule (PECAM) 1 on the endothelial surface. These members of the immunoglobulin superfamily and integrins mediate also the penetration of leucocytes into the arterial wall. (Reviewed e.g. in Huo & Ley 2001.)

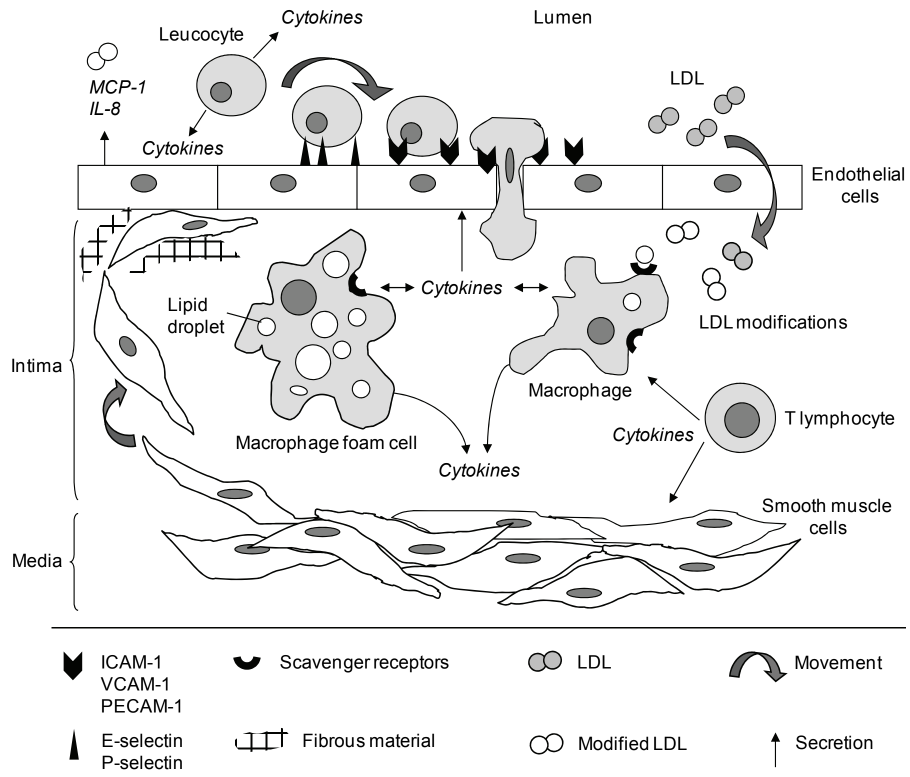

Fig. 5. Several cell types are involved in the development of an arterial lesion. Leucocyte and smooth muscle cell (SMC) migration into the intima and inflammatory processes are key features in the development of atherosclerosis. ICAM-1, intercellular adhesion molecule 1; IL, interleukin; LDL, low density lipoprotein; MCP-1, monocyte chemoattractant protein 1; PECAM-1, platelet-endothelial cell adhesion molecule 1 TNFα, tumor necrosis factor alpha; VCAM-1, vascular cell adhesion molecule 1.

Inside the arterial intima, monocytes differentiate into macrophages that can take up modified lipids through scavenger receptors, eventually becoming converted into foam cells fulled with cholesterol formed lipid droplets. The inflammatory activation inside the arterial wall stimulates migration and proliferation of SMCs inside the intima. SMCs produce extracellular matrix proteins that eventually form a fibrous cap on top of the lesion. (Reviewed in Glass & Witztum 2001.) The clinical manifestations of atherosclerosis are usually caused by the rupture of the fibrous cap. Rupture does not invariably lead to infarction or stroke but on occasions, it can cause stenosis and growth of the lesion. Macrophages are believed to enhance the disruption of the lesion e.g. by production of MMPs that degrade the building material of the fibrous cap. (Reviewed by Libby 2002.)

Monocytes and macrophages are prevalent in the atherosclerotic lesion and human resistin has also been detected inside lesions (Burnett *et al.* 2005, Jung *et al.* 2006). In an immunohistochemical comparison, a carotid endarterectomy sample exhibited strong staining for resistin compared to a non-atherosclerotic internal mammary artery (Burnett *et al.* 2005). A similar difference was also observed in mouse resistin when comparing arteries of healthy C57BL/6J mice and atherosclerotic ApoE-/- mice (Burnett *et al.* 2005). Moreover, in human aneurysms, resistin protein was reported to co-localize with CD68 positive macrophages (Jung *et al.* 2006). In this experiment resistin was not detected with immunohistochemistry in normal arteries or varicose veins (Jung *et al.* 2006). Though aneurysms are not the most representative phenomena of atherosclerosis, these results show that resistin is present in the arterial wall, where there are regions of inflammation.

2.4.1 Resistin promotes proatherogenic changes in arterial wall

Resistin circulates in blood and is expressed in lesion macrophages making it closely linked to other cell types in the lesion. Indeed resistin has been shown to promote changes in endothelial cells and SMCs (Fig. 3). It has been reported that resistin can induce the expressions of adhesion molecules. Human resistin has been shown to up-regulate VCAM-1 both at the mRNA (Kawanami *et al.* 2004) and the protein level (Kawanami *et al.* 2004, Skilton *et al.* 2005, Verma *et al.* 2003). In addition, resistin has been detected to activate MCP-1 secretion (Burnett *et al.* 2005, Verma *et al.* 2003) and mRNA expression (Takata *et al.* 2008) in different human endothelial cells. Resistin has also been reported to increase the expressions of ICAM-1 and long pentraxin 3, a marker of inflammation, in

human aortic endothelial cells (Kawanami *et al.* 2004). Furthermore, in population based studies, it has been shown that the plasma resistin concentration correlates positively with that of soluble VCAM-1 (Hui-Bing *et al.* 2006, Pilz *et al.* 2007), ICAM-1 (Harsch *et al.* 2004, Pilz *et al.* 2007, Reilly *et al.* 2005), and MCP-1 (Aquilante *et al.* 2008). However, in human umbilical vein endothelial cells (HUVECs), exposure of the cells to resistin failed to induce the cell surface expressions of ICAM-1 or E-selectin (Skilton *et al.* 2005). In the same study, Skilton and co-workers reported that resistin did not affect monocyte adhesion to endothelial cells though it was able to up-regulate VCAM-1 (Skilton *et al.* 2005).

Resistin has been shown to enhance the expression of several other endothelium modifying factors such as endothelin-1 (ET-1) (Jung *et al.* 2006, Verma *et al.* 2003), plasminogen activator inhibitor-1 (PAI-1) (Jung *et al.* 2006), vascular endothelial growth factor receptors (VEGFR)-1 and 2, as well as MMP-1 and 2 (Mu *et al.* 2006) (Fig. 3). The plasma ET-1 concentration has also been reported to correlate with the plasma resistin level (Hu *et al.* 2007b). VEGFRs and MMPs are involved in angiogenesis and indeed resistin has been reported to induce proliferation, migration, and capillary-like tube formation of human coronary artery endothelial cells (Mu *et al.* 2006). Furthermore, it has been claimed that human resistin can cause endothelial dysfunction in porcine coronary arteries through oxidative stress and down-regulation of nitric oxide synthase (Kougias *et al.* 2005).

Resistin has been reported to influence also SMCs (Fig. 3). In human aortic SMCs, resistin was detected to promote proliferation (Calabro *et al.* 2004) and in freshly isolated vascular SMCs to increase migration (Jung *et al.* 2006). Both of these changes are part of the development of the fibrous atherosclerotic lesion (Ross 1999). Though resistin is produced by PBMCs, it can also affect macrophages. Resistin has been detected to induce changes in macrophages that are part of the foam cell formation. Resistin has been shown to increase cholesterol esterification, as well as triglyceride and total cholesterol mass in THP-1 macrophages, cells that themselves do not express resistin (Rae & Graham 2006, Rae *et al.* 2007). The increase of lipid accumulation by resistin has been proposed to be mediated through up-regulation of scavenger receptor CD36 but not macrophage scavenger receptor (SR-A) cell surface expression (Xu *et al.* 2006). One interesting question is whether resistin can affect cholesterol removal from the cells but this still waits for clarification.

2.4.2 Resistin in cardiovascular disease

Cell culture studies (Fig.3) have indicated that resistin could play some role in the development and progression of atherosclerosis. The study of Reilly and co-workers (2005) was the first to clarify this relationship in a population based study. They examined the association between plasma resistin and CAC in a CAD symptom free population of 879 subjects with a family history of premature calcification (Reilly *et al.* 2005). A significant association between resistin and increasing CAC (odds ratio of 1.23 with 95% confidence interval of 1.03–1.52) was discovered even after adjusting the results for age, sex, and established risk factors (Reilly *et al.* 2005).

Several case-control and prospective studies have been executed to further clarify the significance of resistin in cardiovascular disease (CVD). The results of these case-control studies are summarized in Table 3. Case-control research frame has also been included in some of the prospective studies described in Table 4. Though these case-control studies include patients with different status of disease, most of them have reported higher plasma resistin concentrations in CVD patients compared to healthy subjects. In addition, studies with further division according to the severity of the disease have shown difference in the concentration of resistin (Hu *et al.* 2007b, Lubos *et al.* 2007, Pilz *et al.* 2007, Qiao *et al.* 2007). The plasma level of resistin was reported to be higher in the patients with more acute types of CAD such as acute MI and unstable angina pectoris compared to stable CAD cases (Hu *et al.* 2007b, Lubos *et al.* 2007, Pilz *et al.* 2007, Qiao *et al.* 2007).

Table 3. Association of plasma/serum resistin concentration to cardiovascular diseases in case-control studies.

Author	Study population	Result
Al-Daghri *et al.* 2005	Cases: 50 subjects with coronary heart disease Controls: 22 healthy controls	Cases had 1.3-fold higher serum resistin levels than controls.
Baker *et al.* 2006	Cases: 46 CAD patients undergoing CABG Controls: 38 healthy controls	Cases had higher serum resistin levels than controls.
Burnett *et al.* 2005	Cases: 39 CAD patients proven by angiography or MI before age 45 Controls: 38 controls over age 45 without evidence of stenosis	Cases had higher plasma resistin levels than controls.
Burnett *et al.* 2006	Cases: 100 subjects with previous MI Controls: 100 controls without CV disease	Cases had higher plasma resistin levels than controls.
Hu *et al.* 2007b	Cases: 46 patients with UAP and 37 with SAP Controls: 31 control without CV disease	Cases had higher plasma resistin levels than controls as well as UAP compared to SAP group.
Ohmori *et al.* 2005	Cases: 157 with CAD (at least one stenosis ≥ 50%) Controls: 73 individuals without CAD	Cases had higher serum resistin levels than controls.
Pischon *et al.* 2005	Cases: 185 women with CAD (verified by angiography) Controls: 227 women without CAD	Cases had higher plasma resistin levels than controls.
Qiao *et al.* 2007	Cases: 24 acute MI patients and 19 with UAP and 22 with SAP Controls: 26 control without CV disease	Cases had higher serum resistin levels than controls as well as acute MI group compared to UAP and SAP as well as UAP compared to SAP.
Yaturu *et al.* 2006	Cases: 57 CAD patients Controls: 45 healthy controls	No difference between cases and controls

CABG, coronary artery bypass grafting; CAD, coronary artery disease; CV, cardiovascular; MI, myocardial infarction; SAP, stable angina pectoris; UAP, unstable angina pectoris.

The largest prospective study of Lubos and colleagues (Table 4) with 1888 cases with more than 30% stenosis in at least one major coronary artery showed that plasma resistin level was associated with future cardiovascular (CV) death when adjusted for age and sex (Lubos *et al.* 2007). When further adjustments for BMI, hypertension, diabetes, smoking, LDL, extent of the disease and therapy were performed, each increase of one standard deviation in plasma resistin level was associated with a hazard risk ratio (HR) of 1.22 (95% confidence interval 1.04–1.43, $P < 0.05$) for future CV death (Lubos *et al.* 2007). In the same study, Lubos and colleagues reported that in patients with acute coronary syndrome resistin

increased within 3–6 hours after the onset of chest pain, suggesting that a high resistin level could represent the inflammatory process preceding myocardial necrosis (Lubos *et al.* 2007).

However, not all of the follow-up studies (Table 4) have detected any association between plasma resistin concentration and future cardiovascular incidents. Some of these studies may lack power to detect these kinds of association because of the low number of participants. It needs to be clarified whether the plasma resistin level is a marker of inflammatory reaction in acute cases of infarction and whether it has a predisposing role for future CV events.

Table 4. Prospective studies on the association of plasma resistin concentration with cardiovascular disease.

Author	Study population	Follow-up time (years)	Result from case-control comparison	Risk (HR with 95% confidence interval if reported)
Efstathiou *et al.* 2007	Follow-up: 211 subjects with atherothrombotic ischemic stroke, 101 deaths	5		Resistin was independent predictor of mortality [HR 2.12 (1.31–5.08, $P < 0.001$)].
Hoefle *et al.* 2007	Cases: 452 with CAD (visible lumen narrowing) Controls: 95 individuals (absence of visible lumen narrowing) Follow-up: All subjects, 113 CV incidents	4.0	No difference between cases and controls. Within the cases no difference between groups of any narrowing and significant stenosis ≥ 50%.	No association with incidence of coronary events
Liang *et al.* 2008	Cases: 55 with progression in CAD Controls: 102 no visible progression of CAD Follow-up: 157 subjects with angiographic report	5	No difference between cases and controls.	Not reported
Lim *et al.* 2008	Follow-up: 343 T2D, 38 CV incidents	2		No association with incidence of coronary events
Lubos *et al.* 2007	Cases: 1888 with CAD (at least one stenosis > 30% in major artery) Follow-up: All subjects with CAD, 70 CV deaths	2.6 (max 5.0)	Plasma resistin levels were higher in patients with acute angina at rest, NSTEMI, and STEMI compared to stable angina pectoris groups.	Unadjusted rate of CV death was highest in the third resistin tertile. HR [1] for one SD increase in resistin level was 1.33 (1.15–1.55, $P < 0.001$) for future CV death.
On *et al.* 2007	Cases: 45 T2D with CAD Controls: 47 T2D Follow-up: 70 T2D with CAD undergone stent implantation	0.5	Serum resistin levels were higher in cases than controls	Pre-implantational serum resistin levels were higher in the subjects with restenosis compared to non-restenotic group
Pilz *et al.* 2007	Cases: 911 with CAD (at least one stenosis ≥ 20%) Controls: 251 individuals with stenosis < 20% Follow-up: All subjects, 133 CV deaths	5.47	No difference between cases and controls but cases with unstable CAD [2] had higher plasma resistin levels than stable CAD cases.	2.33 (1.43–3.80, $P = 0.001$) HR for CV death for fourth compared to first resistin quartile. NS after adjustments.

[1] Adjusted for age and sex. HR remained significant after further adjustments for risk factors. [2] Unstable CAD: UAP, NSTEMI, or STEMI. CAD, coronary artery disease; CV, cardiovascular; HR, hazard ratio; MI, myocardial infarction; NS, not significant; NSTEMI, non-ST-elevation MI; SD, standard deviation; STEMI, ST-elevation MI; T2D, subjects with type 2 diabetes.

2.5 Obesity, type 2 diabetes and resistin

Obesity and diseases associated with it such as type 2 diabetes have become a growing problem in the world (Kahn *et al.* 2006). Obesity is described by an excess amount of white adipose tissue, which stores excess energy in the form of triacylglycerol. In obesity, both the number and size of adipocytes are increased. (Gregoire *et al.* 1998.) Today adipose tissue is considered to be an endocrine organ instead of a simple fuel store (Ahima & Flier 2000). Adipose tissue taken from obese individuals produces elevated amounts of non-esterified fatty acids (NEFAs), glycerol, hormones, cytokines, and growth factors that predispose obese subjects to insulin resistance. Insulin resistance is a condition where tissues do not react normally to insulin. It is associated with both obesity and type 2 diabetes. Since the response to insulin is lowered in target tissues, pancreas β-cells produce more insulin to compensate for the decrease in glucose uptake by the tissues. In type 2 diabetes, the β-cells are no longer able to produce the required amount of insulin to sustain normal glucose tolerance. (Reviewed by Kahn *et al.* 2006.)

In obesity, adipose tissue becomes inflamed and several pro-inflammatory, acute phase, as well as pro-coagulant proteins have been reported to be up-regulated (reviewed recently by Ferrante 2007). Indeed, PBMCs in adipose tissue of obese subjects exist in a pro-inflammatory state (Ghanim *et al.* 2004). In addition, the adipose tissue of obese mice was observed to have been infiltrated with an increased number of macrophages (Xu *et al.* 2003) and it has been reported that the number of macrophages inside the human and mice adipose tissue is increased with BMI and the size of adipocytes (Weisberg *et al.* 2003).

It has been speculated that also type 2 diabetes could represent an inflammatory disease. It has been reported that inflammation predicts the development of both obesity and type 2 diabetes. Furthermore, a higher blood white cell count has been associated with insulin resistance as well as increased levels of acute-phase proteins. (Reviewed in Fernández-Real & Pickup 2008.) Dandona and colleagues (2004) have suggested that the increased glucose and macronutritients could cause oxidative stress and inflammatory changes and the interruption of insulin signaling in insulin resistance would prevent insulin from exerting its anti-inflammatory properties. In addition, the TNFα and IL-6 produced by tissue macrophages could induce insulin resistance by blocking insulin signaling. (Reviewed by Dandona *et al.* 2004.) It has also been speculated that type 2 diabetes is a disorder of innate immune system i.e. the system cannot

cope appropriately with continuous a threat to well-being e.g. overnutrition and inactivity (Fernández-Real & Pickup 2008).

2.5.1 Resistin and obesity

Adiponectin and leptin that are produced by adipocytes and their plasma concentrations are clearly linked to obesity (reviewed e.g. by Meier & Gressner 2004). In the case of resistin, the studies have shown a considerable amount of discrepancies. In cell culture studies, adipocytes have been shown react to resistin (Fig. 3). Ort and colleagues (2005) stated that resistin increased proliferation of preadipocytes as well as triglyceride lipolysis. These authors speculated that this might mean that adipocytes would contain smaller lipid droplets (Ort *et al.* 2005). On the other hand, with a longer exposure time, McTernan and co-workers did not detect changes in the accumulation of lipids or lipolysis in adipocytes (McTernan *et al.* 2003). Nagaev and colleagues reported that resistin did not promote changes in the expression of adipose-specific factors C/EBPα, fatty acid binding protein 4, or glucose transporter (GLUT) 4 in adipose tissue (Nagaev *et al.* 2006).

Population based studies have not clarified the role of resistin in obesity. No correlation with BMI has been reported for example in a general population of 1090 subjects without medication recruited for a study of intima-media thickness (IMT) progression (Norata *et al.* 2007b) or in subjects with type 2 diabetes (n = 199) (Youn *et al.* 2004). In addition, in two large studies of 1922 and 1162 patients with or without CAD, resistin was not correlated with BMI (Lubos *et al.* 2007, Pilz *et al.* 2007). In middle aged women (n = 123) and in young subjects (n = 120), the plasma resistin level was not associated with body fat mass or BMI (Lee *et al.* 2003). Furthermore, when studying normal weight, obese, and obese diabetic groups Heilbronn and co-workers (Heilbronn *et al.* 2004) did not detect any link between the plasma resistin concentration and BMI, percent body fat, intrahepatic fat or fat cell size.

However, an association between resistin and obesity has been reported in almost as many papers as in those where no association was seen. The plasma resistin concentration has been associated with higher BMI in subjects with type 2 diabetes (n = 113) (Tokuyama *et al.* 2007), nondiabetic subjects (n = 123) (Aquilante *et al.* 2008), and asymptomatic subjects with family history of premature CAD (n = 879) (Reilly *et al.* 2005). A correlation between plasma resistin level and body fat mass or percent body fat has been reported in young

healthy subjects (Yannakoulia *et al.* 2003), patients positive for human immunodeficiency virus (HIV) (Barb *et al.* 2005), as well as in overweight Pima Indians, where resistin was also associated with the increase in percent body fat during an average of 4.5 year follow-up (Vozarova de Courten *et al.* 2004). Furthermore, the plasma resistin concentration has been shown to be positively correlated with total extremity and abdominal subcutaneous but not with abdominal visceral fat in HIV positive individuals (Barb *et al.* 2005). However, these correlations in HIV patients disappeared when sex was taken into account (Barb *et al.* 2005). In contrast, in Japanese subjects with type 2 diabetes, the plasma resistin level was associated with visceral but not subcutaneous fat area also in both sexes separately (Matsuda *et al.* 2004).

In weight loss studies, plasma resistin concentration have usually reacted to weight loss similarly as other markers of inflammation (TNFα, IL-6) with no change (Giannopoulou *et al.* 2005, Monzillo *et al.* 2003) or decrease (Jung *et al.* 2007) in their concentrations. The adipose tissue of obese subjects is known to contain an increasing number of leucocytes as the obesity progresses (Curat *et al.* 2006, Weisberg *et al.* 2003, Xu *et al.* 2003). This could be the reason for the link between resistin and obesity that has been reported in several studies since resistin may be produced by adipose tissue macrophages (Curat *et al.* 2006).

2.5.2 Plasma resistin in type 2 diabetes

Originally resistin was reported to impair insulin sensitivity in wild type mice (Steppan *et al.* 2001a). The human cell culture studies which have been summarized in Fig. 3 have not presented convincing results on the effects of resistin on diabetes related phenomena. The hepatocytic cell line HepG2 has been reported to react to resistin by decreasing activation of insulin signaling leading to increasing levels of insulin resistance (Zhou *et al.* 2007). In another human hepatocyte cell line, L-02, resistin was further shown to impair insulin stimulated glucose utilization by changing the expression of several glucose metabolism and insulin signaling pathway genes (Zhou *et al.* 2006). The expression of GLUT2 was not altered (Zhou *et al.* 2006) which is in accordance with GLUT4 results obtained from adipocytes (Nagaev *et al.* 2006). In conclusion, hepatocyte studies suggest that resistin could impair the actions of insulin in liver whereas studies from adipocytes have shown that in adipocytes, incubation with resistin decreased glucose uptake (McTernan *et al.* 2003) but did not alter insulin-stimulated glucose uptake (Ort *et al.* 2005).

Studies on the plasma resistin level in type 2 diabetes have described similarly conflicting results as the studies on obesity. There are studies that have reported higher plasma resistin concentrations in subjects with type 2 diabetes (Al-Daghri *et al.* 2005, Al-Harithy & Al-Ghamdi 2005, Dullaart *et al.* 2007, Fujinami *et al.* 2004, Hasegawa *et al.* 2005, Hui-Bing *et al.* 2006, Koçak *et al.* 2007, Lu *et al.* 2006, McTernan *et al.* 2003, Takata *et al.* 2008, Youn *et al.* 2004, Zhang *et al.* 2003) whereas there is an almost equal number of studies in which no such difference has been observed (Chen *et al.* 2006, Fehmann & Heyn 2002, Heilbronn *et al.* 2004, Lee *et al.* 2003, Norata *et al.* 2007b, Stejskal *et al.* 2003, Yaturu *et al.* 2006). A common limitation of all of these studies is the low number of subjects in both case and control groups. The study of Youn and colleagues was the only one with more than 100 individuals in both groups (Youn *et al.* 2004). Only one study has reported that the plasma resistin level is lower in the group of type 2 diabetics (n = 555) compared to healthy controls (n = 216) (Schäffler *et al.* 2004).

The association of resistin to diabetes is obscure since even those studies observing a difference in plasma resistin level between subjects with type 2 diabetes and controls have not always detected significant association between resistin and insulin resistance (Hasegawa *et al.* 2005, Koçak *et al.* 2007, Youn *et al.* 2004). Furthermore, the largest studies (n > 500) on plasma resistin concentration with the exception of the study of Pilz and co-workers (Pilz *et al.* 2007), have not detected any independent association with insulin resistance in different study populations (Hoefle *et al.* 2007, Norata *et al.* 2007b, Reilly *et al.* 2005).

Some of the studies that have reported higher plasma resistin level in subjects with type 2 diabetes described also higher levels of other inflammatory markers CRP, TNFα, or IL-6 (Dullaart *et al.* 2007, Hasegawa *et al.* 2005, Koçak *et al.* 2007). In a prospective, placebo-controlled, double-blinded study of the effects of rosiglitazone treatment for 12 weeks in subjects with metabolic syndrome it was noted that the decrease in the plasma resistin concentration correlated with the changes in IL-6 and soluble TNFα receptor 2 (Samaha *et al.* 2006).

Comparative studies of rosiglitazone and metformin treatments on type 2 diabetes have shown that rosiglitazone but not metformin was able to decrease the plasma resistin concentration, suggesting that the decrease in resistin was not a result of improved insulin resistance since both treatments decreased insulin resistance but rather, was a consequence of the anti-inflammatory properties of rosiglitazone (Jung *et al.* 2005, Kim *et al.* 2007). Taken together it can be

speculated that the higher levels of resistin in diabetes could reflect a state of inflammation rather than a causative link to type 2 diabetes and insulin resistance.

3 Aims of the study

This study was intented to clarify the role of resistin in humans using genetic, epidemiological and cell culture methods. The aims and specific study questions in each original work were:

I To study the connection between resistin and type 2 diabetes.

- Do genotype or allele frequencies of *RETN* SNPs differ between patients with type 2 diabetes and nondiabetic control population?
- Do *RETN* SNPs associate with clinical parameters related to diabetes?

II To study the connection of *RETN* variants with the plasma resistin concentration.

- Does the plasma resistin level differ between genotypes of *RETN* SNPs?
- Do genotype or allele frequencies of *RETN* SNPs differ between low and high atherosclerosis risk groups?

III To study the association of resistin with atherosclerosis.

- Does the plasma resistin concentration associate with carotid artery IMT which is a surrogate marker of early atherosclerosis?
- Do risk factors of atherosclerosis act as determinants of the plasma resistin level?

IV To study the role of resistin in cells related to atherosclerosis

- Which white blood cell types express resistin?
- Is expression of resistin in different white blood cell types modified by differentiation or inflammatory factors such as LPS and TNFα?
- Does resistin promote proatherogenetic changes in endothelial cells?

4 Subjects and methods

4.1 Study populations

In the original papers I, II and III, previously defined study populations were used. Here only the principal characteristics of these study populations will be described, since they have been depicted in more detail in the original papers of this thesis. The studies were approved by the Ethical Committee of the Faculty of Medicine, University of Oulu and informed consent was obtained from all subjects.

4.1.1 Study group of subjects with type 2 diabetes (I)

This study group was collected during the years 1988–90 originally for the study of the association of genetic polymorphisms in lipid regulatory proteins with micro- and macroangiopathy in patients with non-insulin-dependent diabetes mellitus. The study population consisted of 271 subjects, who came to Oulu University Hospital for evaluation of their diabetes treatment. The study population has been described in detail in the report of Ukkola and co-workers. (Ukkola *et al.* 1993). For the resistin study I, isolated DNA samples were available from 258 subjects, 124 women and 134 men. These study subjects were middle aged (mean age 58 years), obese (mean BMI 28 kg/m^2) with type 2 diabetes, who had poor or unsatisfactory metabolic control. Lipids and lipoproteins, blood glucose, glycosylated hemoglobin A1 (GHb_{A1}), and C-peptide levels as well as the presence of cerebrovascular disease and CVD were determined as described in article I.

4.1.2 OPERA (I–III)

Oulu Project Elucidating Risk of Atherosclerosis (OPERA) evaluated the risk factors and disease end points of atherosclerotic CVD. Shortly, the OPERA study consisted of subjects with high blood pressure confirmed by a verified need for hypertensive drug treatment and the age- and sex-matched control subjects without established high blood pressure. Treated hypertensive subjects were randomly selected from the Social Insurance Institute Register for the reimbursement of hypertension medication and the control population was

randomly selected from the Finnish National Health Register. The original OPERA study group collected during 1990–1993 included 518 hypertensives and 526 control subjects with almost equal proportions of men and women. Study population and lipid, blood pressure, glucose metabolism, and intima-media thickness measurements have been described in detail in earlier reports (Kauma *et al.* 1996, Kiema *et al.* 1996, Päivänsalo *et al.* 1996, Rantala *et al.* 1998).

In the study I, the OPERA control population was used as nondiabetic control group for the cases with type 2 diabetes. For this comparison the individuals with type 2 diabetes were excluded from the control population resulting in 494 (253 females and 241 males) control subjects. The mean age of the controls was 51.3 years and their mean BMI was 26.3 kg/m^2. In study II, both plasma and isolated DNA samples from 510 control subjects and 505 DNA samples from the hypertensive group were examined. In study III, a total of 525 control plasma samples were used. The methods specific for these studies have been described in detail in the original articles II and III.

4.2 Laboratory methods

Several common measurements have been undertaken on the plasma samples of the study populations. These methods are briefly illustrated in Table 5.

Table 5. Methods used to measure common clinical features in plasma.

Clinical feature	Method	Used in original publication
Plasma total cholesterol	Enzymatic colorimetric method	I–III
Plasma triglycerides	Enzymatic colorimetric method	I–III
VLDL cholesterol	Enzymatic colorimetric method from fraction formed during ultracentrifugation.	I–III
HDL cholesterol	Enzymatic colorimetric method from supernatant after precipitation of lower density lipoproteins from fraction formed during ultracentrifugation.	I–III
LDL cholesterol	Calculated by subtracting cholesterol in HDL from the cholesterol concentration of the VLDL free fraction.	I–III
Blood glucose	Hexokinase method	I
	Glucose dehydrogenase method	II, III
Plasma insulin	Commercial RIA	II, III
GHb_{A1}	Commercial agar gel electroendo-osmotic method	I
GHb_{A1c}	Liquid chromatography	II, III
Total IGF-1	Commercial ELISA	II, III
IGFBP-1	Commercial ELISA	II, III
hsCRP	Commercial ELISA	II, III
Leptin	Commercial RIA	II, III

ELISA, Enzyme-linked immunosorbent assay; GHb_{A1}, glycosylated hemoglobin A1; HDL, high density lipoprotein; hsCRP, highly sensitive C-reactive protein, IGF-1, insulin-like growth factor 1; IGFBP-1, IGF binding protein 1; LDL, low density lipoprotein; RIA, Radioimmunoassay; VLDL, very low density lipoprotein,

4.2.1 Measurement of IMT (III)

IMT was measured with the carotid ultrasound procedure as previously described (Päivänsalo *et al.* 2006). In short, IMT determines the distance between the media-adventitia interface and the lumen-intima interface. IMT was measured from the near and far wall of both sides of the carotid artery. The measurements were made on the internal carotid artery, the bifurcation enlargement, and three sites of the common carotid artery. We tested the association with the mean value of the 20 measurements (mean IMT), the mean of the far wall measurement of the common carotid artery (CCA), the bifurcation enlargement (BIF), and the inner carotid artery (ICA).

4.2.2 *Genotyping of RETN SNPs (I and II)*

Three resistin gene SNPs were studied, promoter variant SNP-420C>G and SNP +157T>C and +299G>A in the second intron of *RETN*. All of these three SNPs modify the recognition sequence of some restriction enzyme and the genotyping was based on restriction fragment length polymorphism (RFLP). The areas around the SNPs were multiplied by polymerase chain reaction (PCR) and the PCRs products were digested with restriction enzymes as described in detail in study I. Different genotypes were visualized based on the length of the formed restriction fragments. A sample from every genotype was carried in every restriction reaction to confirm the success of the reaction.

4.2.3 *Measurements of resistin concentration (II–IV)*

Two methods were used in the measurements of resistin concentration. From the OPERA plasma samples, resistin was measured with a commercial enzyme-linked immunoassay kit (ELISA) (Linco Research Inc., USA) as described in papers II and III. From the cell extracts and growth medium samples of study IV, resistin was measured with an in-house assay based on the DELFIA® technique (PerkinElmer) as described in paper IV. The intra- and interassay coefficients of variation from the in-house assay were 3.9 and 8.4%, respectively. The commercial ELISA kit was based on the enzymatic reaction of horseradish peroxidase, where the absorbance of the formed reaction product was measured, whereas the in-house assay was based on time-resolved fluorometry (DELFIA) where the emitted fluorescence of enhanced europium was measured. The methods were compared with separate plasma samples where the commercial kit gave mean concentration of 7.7 ng/ml and the in-house assay 8.5 ng/ml. The resistin concentrations obtained with the commercial and in-house assay correlated strongly ($r = 0.754$, $P \leq 0.001$) though the in house-assay gave about 10% higher concentrations.

4.2.4 Real-time PCR (IV)

Real-time PCR (RT-PCR) was used to monitor expression levels of several genes. In these experiments, total RNA was separated from cell samples and mRNAs were reverse transcripted with poly-A-tail corresponding primers to DNA as described in original paper IV. RT-PCR was done with Syber Green chemistry (BioRad), where the amount of PCR product generated is comparable to the increase of fluorescence produced by SYBR Green when bound to double-stranded DNA. In the RT-PCR, every sample was determined in duplicate or triplicate. The expression of target gene in different samples was standardized with the expression of one or two housekeeping genes in the samples. β-actin was used as a reference gene in the white blood cell exposure samples whereas glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference in EA.hy 926 samples. When comparing resistin expression in different cell types, the results were normalized to both β-actin and GAPDH. The RT-PCR results are presented as fold changes compared to control sample.

4.2.5 Primary and immortal cells in culture (IV)

In article IV, several immortal cell lines were used and they are described in Table 6 with information of the experiments in which they were used. These experiments have been described in detail in the original paper. All the cell culture exposures were conducted with at least three replicate wells and repeated a minimum of three times. To assure that cells were in an active dividing state, cells were not grown to confluency before the experiments.

Table 6. Description of the cell lines used.

Cell line	Definition	Experiments
COS-7	SV40 transformed African green monkey kidney fibroblast like cell line (Gluzman 1981).	Transfected for secretion of resistin into medium
EA.hy 926	Formed by hybridization of human umbilical vein endothelial cells with lung carcinoma cell line A549 (Edgell *et al.* 1983).	To study the monocyte adhesion to endothelial cell monolayer and adhesion molecule expression.
HL-60	Human myeloblast cell line isolated from patient with acute promyelocytic leukemia. Can be differentiated towards monocytic, granulocytic, and eosinophilic lineages. (Reviewed by Harris & Ralph 1985.)	To study the expression of resistin at myeloblast state and after differentiation into neutrophils.
Jurkat (clone E6-1)	Human T lymphocyte cell line isolated originally from patient with acute T cell leukemia (Weiss *et al.* 1984).	To study the expression of resistin and the effects of LPS and TNFα.
RPMI-8226	Human B lymphocyte cell line derived from a myeloma (Han *et al.* 1979).	To study the expression of resistin and the effect of LPS and TNFα.
THP-1	Human monocytic cell line isolated from patient with acute monocytic leukemia. Can be induced to differentiate into macrophage-like cells. (Reviewed by Auwerx 1991.)	To compare the expression of resistin in different white blood cells and in adhesion assay. To explore the expression of resistin during macrophage differentiation.
U937	Human monoblastic cell line isolated from patient with diffuse histiocytic lymphoma. Can be induced to terminal monocytic differentiation. (Reviewed by Harris & Ralph 1985.)	To study expression of resistin at the myeloblast state and after differentiation into macrophages. To explore the effects of LPS and TNFα on resistin expression.

LPS; lipopolysaccharide, TNFα, tumor necrosis factor alpha.

Separation on human white blood cells

For the study of the expression of resistin in different human leucocytes, blood was collected from ten healthy volunteers. The approval for this study was given by the Ethical Committee of University of Oulu. No personal information from the volunteers was needen in this study. Human white blood cells were separated to PBMC and granulocyte fractions using the Vacutainer™ CTP™ (BD) tubes. MACS® Technology (Miltenyi Biotec) based on antibody coated magnetic micro beads was used to separate different cell types and their purity was evaluated with flow cytometry (FACSort) using fluorochrome-conjugated antibodies. The procedure of cell separation is illustrated in Fig. 6. Every cell type was separated at least three times.

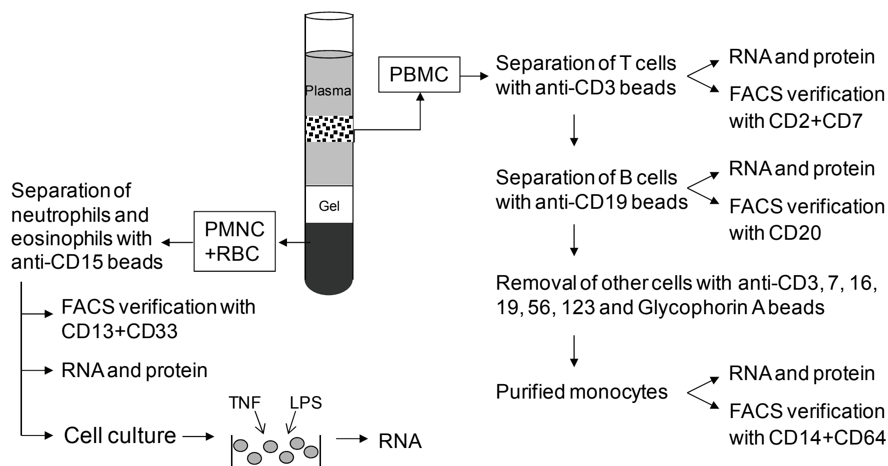

Fig. 6. Separation protocol of human leucocytes. FACS, Fluorescence Activated Cell Sorting; PMNC, polymorphonuclear cells (granulocytes); PBMC, peripheral blood mononuclear cells; RBC, red blood cells; TNF, tumor necrosis factor α; LPS, lipopolysaccharides.

Adhesion assay

Adhesion of mononuclear cells to arterial endothelium was studied using THP-1 monocytes and EA.hy 926 endothelial cell line in the adhesion assay, illustrated in Fig. 7. The method has been described in detailed in article IV.

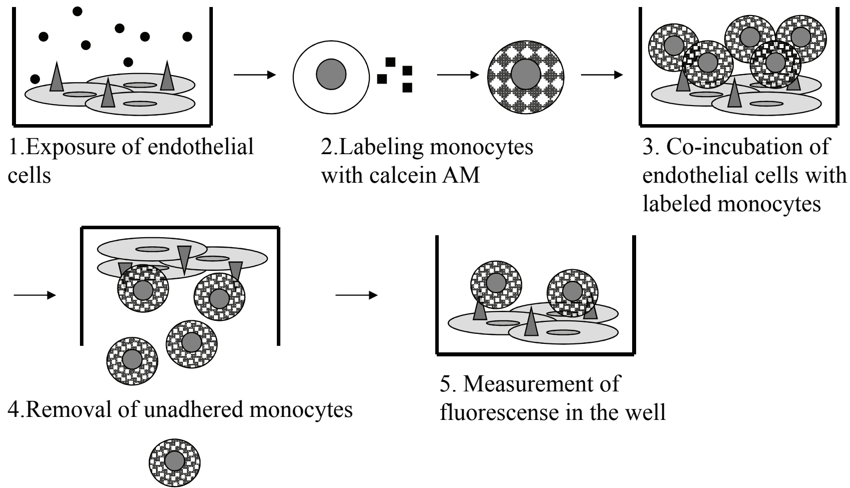

Fig. 7. Illustration of the protocol of adhesion assay. Different steps are shown in one well of the plate.

4.3 Statistical methods (I–IV)

All statistical tests were made with the SPSS software package (© SPSS Inc. Chicago, USA) unless otherwise stated. The *P*-value of 0.05 was considered the limit for statistical significance. The differences in genotype frequencies between the cases and controls and the prevalence of diseases were tested using the χ^2-test. If a difference in the disease prevalence was observed, the association was tested further with binary logistic regression with covariates.

In the cell culture experiments, significance of difference between treated and control samples were tested with independent samples t-test or with Mann-Whitney test depending on the distribution of tested variables. For the clinical continuous variables, logarithm transformation was applied when necessary to normalize the distribution. Correlations were tested with Pearson's Correlation.

OPERA control group was divided into three subgroups, tertiles, according to their plasma resistin level. There were 173 or 174 subjects in each tertile. The analysis of variance (ANOVA) and analysis of covariance (ANCOVA) with adjustments were used to compare the means of continuous variables measured between these resistin tertiles and genotypes. When the interaction between sex and genotype was statistically significant as a predictor of tested variable, the association was tested separately for men and women.

Linear regression was used to test the determinants of the resistin concentration and IMT. Determinants of resistin were tested with the stepping method, in which the probability of F values 0.05 and 0.1 were used as entry and removal of variables, respectively. The enter method was used in the IMT regression model.

5 Results

5.1 *RETN* polymorphisms (I and II)

In the first two studies, the aim was to clarify the associations of human resistin gene (*RETN*) variations. Genotyping of three *RETN* SNPs -420C>G, +157T>C and +299G>A was done with RFLP (Fig. 8).

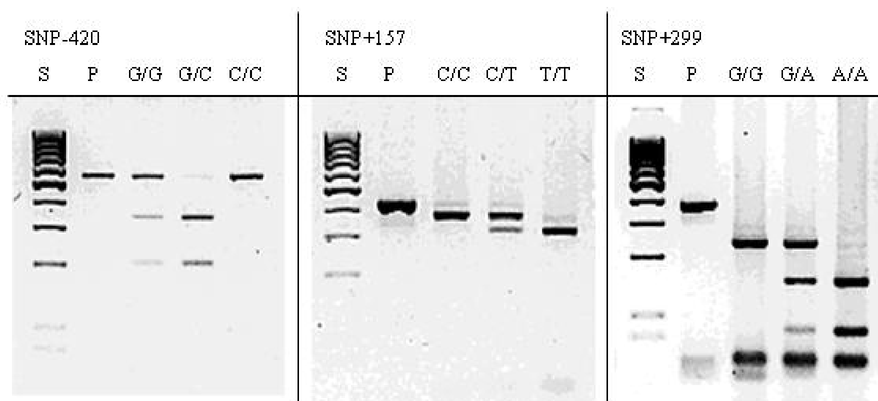

Fig. 8. *RETN* SNPs -420C>G, +157T>C, and +299G>A genotypes based on the variation in the lengths of restriction fragments. In the gel electrophoresis, the wells are identified as: S, nucleotide fragment size standard; P, PCR product before restriction enzyme processing; X/X, different genotypes after restiction reaction.

5.1.1 Subjects with type 2 diabetes and nondiabetic control population do not differ in RETN polymorphisms (I)

In this study, the aim was to explore the association between human resistin and diabetes using a genetic approach. Three *RETN* SNPs -420C>G, +157T>C, and +299G>A were genotyped in the two study populations consisting of subjects with type 2 diabetes and nondiabetic controls. Allele or genotype frequencies of the three SNPs (Fig. 9) did not differ between these study groups. However, in the type 2 diabetes study group, *RETN* SNPs were associated with glucose values. SNP-420C>G and +299G>A were associated with fasting ($P = 0.016$ and $P = 0.041$, respectively) and mean blood glucose (mean of six measurements during 24 h) ($P < 0.001$ and $P = 0.002$, respectively) in men. The SNP+157T>C

association with mean blood glucose was significant in women and men ($P = 0.019$ and $P = 0.005$, respectively). Furthermore, two SNPs, SNP-420C>G and +299G>A, were associated with the prevalence of cerebrovascular disease. In a regression analysis with age, sex, and BMI as covariates, the odds ratios for the rare homozygotes were 8.4 (95% CI 2.4–30.1) and 6.6 (1.9–22.4) compared to the common homozygotes in the SNPs -420C>G and +299G>A, respectively.

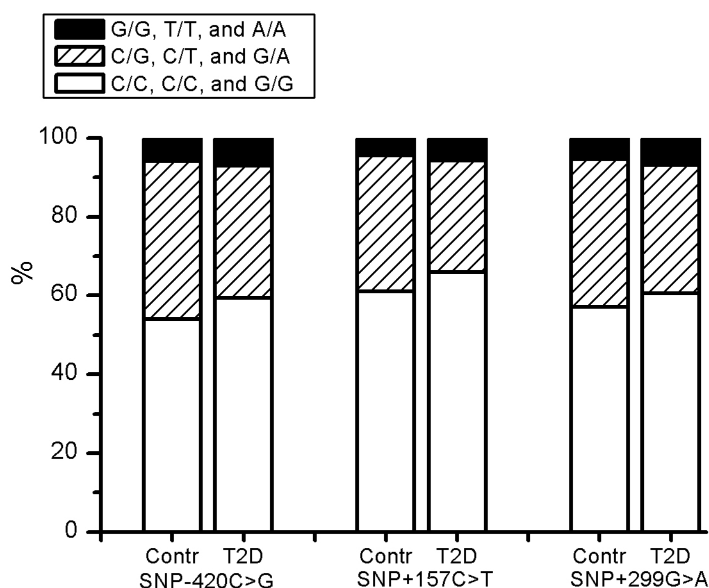

Fig. 9. Genotype frequencies do not differ between nondiabetic control group (Contr) and group of subjects with type 2 diabetes (T2D) in *RETN* SNPs -420C>G, +157T>C, and +299G>A. White colum represents common homozygotes, striped colum heterozygotes, and black colum rare homozygotes in these SNPs.

5.1.2 The common genotypes in RETN SNPs have the lowest plasma resistin concentration (II)

The second aim of this study was to clarify the association of *RETN* variants with the plasma resistin concentration and cardiovascular risk factors in middle-aged subjects in the OPERA study. The data demonstrated that the genotypes of SNP-420C>G, +157T>C, and +299G>A were associated with the plasma resistin concentration ($P = 0.008$, $P = 0.009$, and $P = 0.001$, respectively). Resistin levels

were significantly different (P-values < 0.05) between the genotypes in the three SNPs, even after adjustment for age, sex, number of blood leucocytes, and BMI. The common homozygote had the lowest plasma resistin concentration in all the three SNPs (Fig. 10).

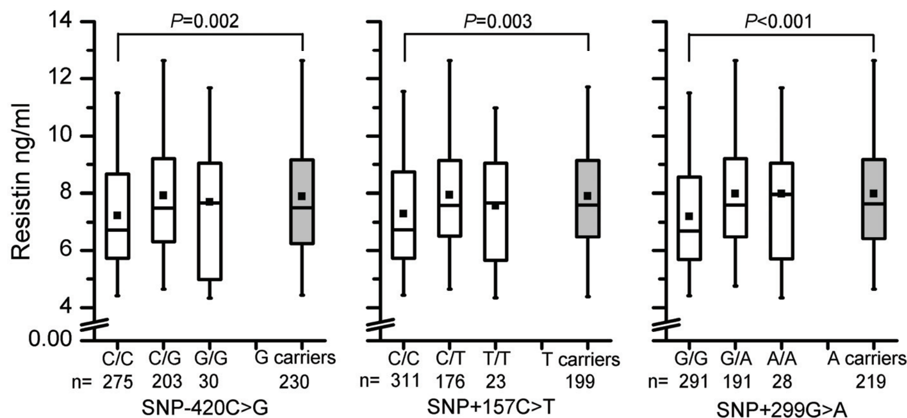

Fig. 10. Plasma resistin concentration differs between genotypes in *RETN* SNP-420C>G, +157C>T and +299G>A. Significance for the difference between common allele homozygotes and rare allele carriers (gray box) is presented. Mean (dot), median (cross line), 25–75% percentile (box), and 5–95% range of the values (whiskers) are presented.

In the OPERA controls, SNP-420C>G was associated significantly with HbA_{1c} ($P = 0.003$), IGFBP-1 ($P = 0.004$), and triglycerides ($P = 0.011$) in both sexes and in insulin ($P = 0.037$) and quick index ($P = 0.029$) in women. After adjustment for age, sex, and BMI, these associations remained significant. However SNP-420C>G was not associated with fasting glucose, number of blood leucocytes or hsCRP.

5.2 Plasma resistin level is associated with inflammatory markers but not with IMT (III)

The third aim of this study was to clarify the association of plasma resistin with atherosclerosis. The first study revealed that SNPs in *RETN* were linked to the prevalence of cerebrovascular disease (I). The OPERA control population was examined for a evaluation of the association of the plasma resistin concentration

with carotid artery IMT, which is a surrogate marker of early atherosclerosis (Lorenz *et al.* 2007). Resistin was not independently associated with IMT measurements when correlations or difference between means in resistin tertiles were analysed. The slight negative correlation between resistin and mean ($r = -0.104$, $P = 0.018$), ICA ($r = -0.099$, $P = 0.025$), and CCA ($r = -0.107$, $P = 0.016$) IMT measurements vanished after controlling for traditional risk factors (age, sex, smoking, BMI, LDL cholesterol, and systolic blood pressure). The correlations between plasma resistin and IMT measuments were nonsignificant when the sexes were analysed separately. The gender-related difference in plasma resistin levels probably caused this detected negative correlation since women had both higher resistin levels and lower IMT values.

A regression analysis was performed to confirm that traditional risk factors are associated with IMT in this study population. In the regression model age ($P < 0.001$), sex ($P < 0.001$), smoking ($P < 0.01$), systolic blood pressure ($P < 0.001$) and LDL cholesterol proved to be associated with mean IMT whereas hsCRP ($P = 0.350$), BMI ($P = 0.238$) and resistin ($P = 0.191$) were not. In total, these factors explained 29.3% (adjusted R^2) of the variation in IMT ($P < 0.001$).

Though we did not detect independent association between plasma resistin concentration and IMT we discovered significant association between resistin and the number of blood leucocytes as well as hsCRP (Fig. 11). The differences between tertiles in the number of leucocytes and hsCRP remained significant even after controlling for age, sex and BMI ($P < 0.001$ and $P < 0.01$, respectively).

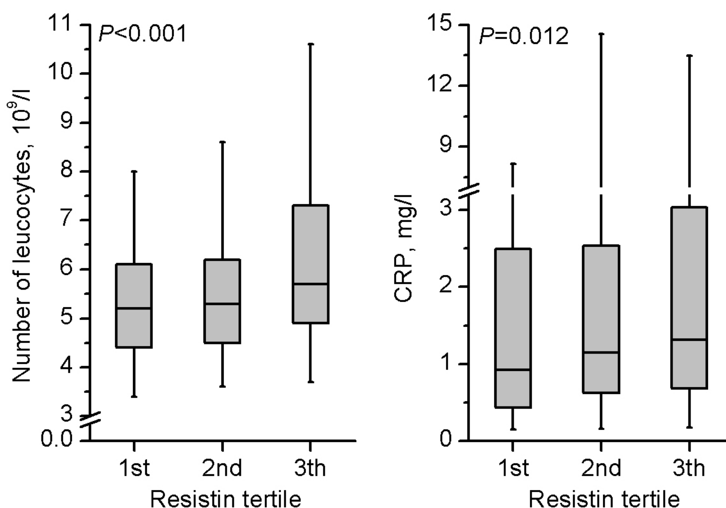

Fig. 11. The number of blood leucocytes and the concentration of highly sensitive C-reactive protein (CRP) differed between the resistin tertiles (tested with analysis of variance). Median (crossline), 25–75% percentile (box), and 5–95% range of the values (whiskers) are presented.

There were significant correlation coefficients between resistin and the number of blood leucocytes ($r = 0.235$, $P < 0.001$) as well as with hsCRP concentration ($r = 0.139$, $P < 0.002$). Both correlations persisted also when the sexes were tested separately. Significant but weaker correlations were detected also between plasma resistin and insulin-like growth factor binding protein 1 (IGFBP-1), hemoglobin A_{1c} (HbA_{1c}), leptin, LDL cholesterol and total cholesterol levels as well as diastolic blood pressure and waist-to-hip ratio (see original article III). However, the resistin concentration was not associated with the insulin level or with quick index {calculated with formula; 1/[log(fasting insulin)+log(fasting glucose)]}, which is a mathematical estimate of insulin resistance (data not shown).

Sex and factors correlating significantly with plasma resistin were analysed with the regression model. In the regression model, leucocytes, IGFBP-1, leptin and total cholesterol were found to be significant determinants of the resistin concentration when stepwise selection criteria were used. Standardized β was 0.269 ($P < 0.001$) for leucocytes, 0.221 ($P < 0.001$) for IGFBP-1, 0.192 ($P < 0.001$) for leptin and −0.097 ($P = 0.026$) for total cholesterol. These factors

explained 14.3% (adjusted R^2) of the variation in the plasma resistin concentration.

5.3 Resistin is expressed in different leucocyte populations and it promotes atherosclerotic changes in endothelial cells (IV)

The number of blood leucocytes was observed to be a significant explanatory factor for the plasma resistin concentration in the OPERA study (original article III). Since the expression of resistin in different primary human white blood cell populations had not been studied in detail, the expression of resistin in several primary human white blood cells was next investigated. Separations of highly purified neutrophils+eosinophils (NE), T cells, and monocytes were successful. However, the separation of B cells was not successful enough to obtain a reliable pure fraction for resistin expression studies. The expression of resistin was detected in all separated pure human blood primary cells and white blood cell lines except for NK-92 natural killer cells. The expression level in THP-1 cells was barely detectable and it was used as a reference for baseline expression when relative expressions were calculated (Table 7).

Table 7. Relative expression of resistin at the mRNA and protein level in separated human primary leucocyte populations and in immortalized cell lines.

Cell type	Baseline expression		LPS exposure			TNFα exposure		
			mRNA		Protein	mRNA		Protein
	mRNA	Protein	2 h	24 h	24 h	2 h	24 h	24 h
Monocytes (THP-1)	1	1						
Monocytes (U937)	924	58	↑	↑	↑	↑	↓	↑
Primary monocytes	733	16						
Primary NE	106	2161	↑	↑	–	↑	↑	–
B cells (RPMI-8226)	77	26	↔	↔	↔	↔	↑	↑
HL-60	34	20						
T-cells (Jurkat)	23	47	↔	↔	↔	↑	↓	↓
Primary T cells	13	27						

Results have been compared to the expression in THP-1 cell line and presented as fold changes. In addition, the effects of LPS and TNFα on the expression of resistin in tested cell types are listed. ↑, increase in expression; ↔, no change in expression; ↓, decrease in expression; LPS, lipopolysaccharides; NE, primary neutrophils and eosinophils; TNF, tumor necrosis factor.

Two inflammatory agents LPS and TNFα, have been reported to induce resistin expression in PBMC (Bokarewa *et al.* 2005, Kaser *et al.* 2003, Lu *et al.* 2002, Sundén-Cullberg *et al.* 2007). Since resistin expression was detected in all leucocyte types studied (Table 7) and not only in PBMCs, their responses to LPS and TNFα were compared. Three immortalized cell lines U937, Jurkat, and RPMI-8226 were used to substitute for monocytes, T, and B cells, respectively, that were too few in number to permit culturing. TNFα induced significant changes in resistin expression in all cell lines whereas LPS was only effective in neutrophils and U937 cells (Table 7).

Since monocytes expressed resistin, the change in expression during macrophage differentiation was studied. In the monocytic U937 cells, mRNA (−1.8 fold), intra-cell (−2 fold) and secreted (−1.4 fold) resistin levels all displayed significant declines after 3 days of macrophage differentiation.

Resistin increases monocyte adhesion to endothelial cells

EA.hy 926 and THP-1 cells were used in the adhesion assay because they were observed not to express resistin and in this way the influence of an intra-cellular protein could be ruled out. A dose dependent increase in monocyte adhesion to endothelial cells with human recombinant resistin was observed (Fig. 12). The increase in adhesion by resistin treatment was not influenced by the antibiotic polymyxin B ($P > 0.05$), ruling out any effect of endotoxins in the recombinant protein.

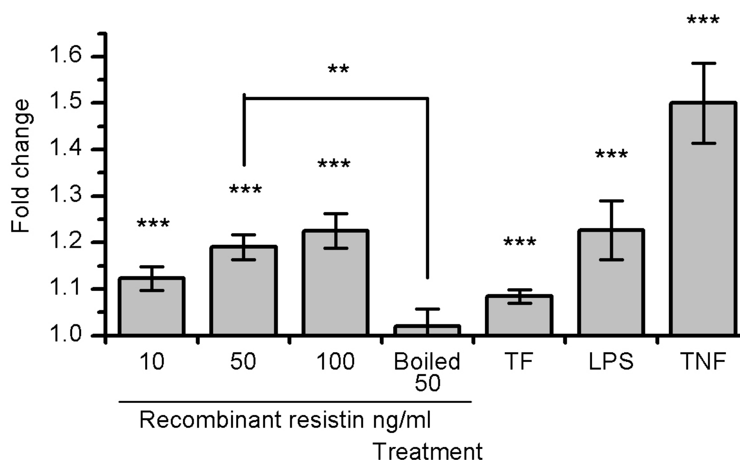

Fig. 12. Resistin increased the adhesion of THP-1 monocyte to EA.hy 926 endothelial cells dose dependently. Results (mean ± standard error of the mean) have been compared to phosphate buffered saline (PBS) control except in the experiments with boiled 50 ng/ml resistin that was compared to unboiled and TF treatment that was compared to the medium of plasmid DNA transfected cells. Endothelial cells were treated for 24 h with resistin and LPS (10 ng/ml) and for 4 h with TNFα (1 ng/ml). TF, medium from resistin (RETNpcDNA) transfected COS-7 cells. * $p \le 0.05$, ** $p \le 0.01$, *** $p \le 0.001$.

In an attempt to clarify the mechanism behind the increase in adhesion, we investigated the changes in the expressions of adhesion molecules and proliferation of EA.hy 926 cells. Resistin increased the RNA expression of MCP-1 and PECAM-1 and decreased the mRNA level of ICAM-1 (Fig. 13). Resistin did not affect significantly the expression of P-selectin. VCAM-1 or E-selectin could not be detected reliably with RT-PCR in untreated or resistin treated EA.hy 926 cells.

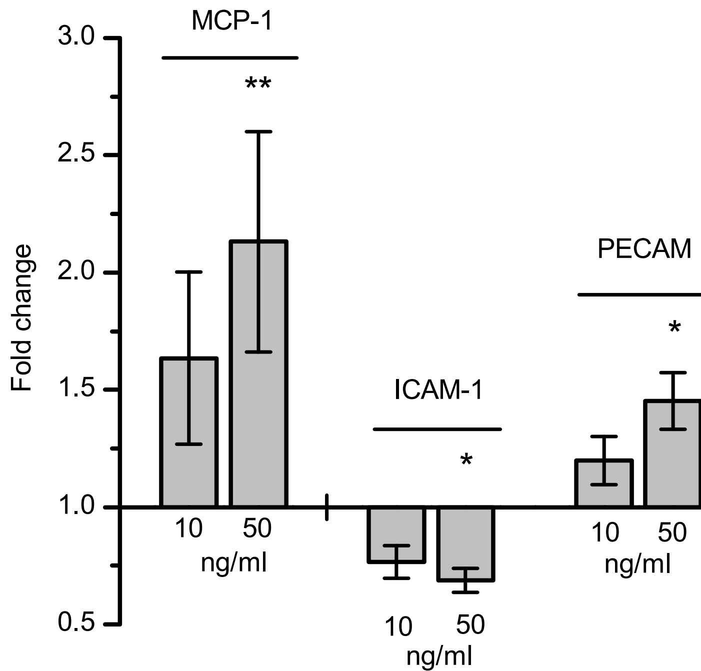

Fig. 13. Exposure to recombinant resistin (50 ng/ml) for 4 hours had a significant impact on the mRNA expression of MCP-1, ICAM-1, and PECAM-1. The results are presented as mean fold changes (± standard error of the mean) compared to phosphate buffered saline (PBS) control. ICAM-1, intracellular adhesion molecule-1; MCP-1, monocyte chemotactic protein-1; PECAM-1, platelet-endothelial adhesion molecule 1.

6 Discussion

6.1 *RETN* SNPs and resistin concentration in plasma

One of the aims of this study was to find out whether genetic variants in the *RETN* were associated with the concentration of resistin in human plasma. Our results in study II showed that the plasma resistin concentration differed significantly between the genotypes in *RETN* SNPs. In our study, the common homozygotes in SNPs -420C>G, +157T>C, and +299G>A had the lowest plasma resistin concentration, even after adjustment for age, sex, number of blood leucocytes, and BMI. In accordance with other studies (Axelsson *et al.* 2006, Azuma *et al.* 2004, Cho *et al.* 2004, Osawa *et al.* 2004, Osawa *et al.* 2005, Osawa *et al.* 2007, Qasim *et al.* in press, Yamauchi *et al.* 2008), the G-allele carriers had higher plasma resistin levels than non-carriers. Several studies have shown that the *RETN* promoter carrying the G allele in the SNP-420C>G is more active than the C form of the promoter (Azuma *et al.* 2004, Cho *et al.* 2004, Chung *et al.* 2005, Osawa *et al.* 2004, Smith *et al.* 2003). No information about their functional effects has been published about the other two SNPs studied. Since there was a clear linkage disequilibrium between the three polymorphisms, it is possible that the associations are due to SNP-420C>G or some other functional variant nearby.

PBMCs are the main producers of resistin (e.g. Nagaev *et al.* 2006, Xu *et al.* 2007), however, adjustment for the number of blood leucocytes did not abolish the association between *RETN* SNPs and the plasma resistin level. SNP-420C>G was not associated with leucocytes in the same study population. Therefore, it can be proposed that the influence of the SNP-420C>G on resistin promoter activity may be one of the factors influencing plasma resistin concentration in humans.

6.2 Leucocyte-derived resistin in atherosclerosis

Atherosclerosis has been considered as an inflammatory disease (reviewed by Libby 2002). Since resistin has been shown to be up-regulated in blood leucocytes by inflammatory factors (e.g. Bokarewa *et al.* 2005, Kaser *et al.* 2003, Lehrke *et al.* 2004) and to produce changes in arterial wall cells (e.g. Calabro *et al.* 2004, Kawanami *et al.* 2004, Verma *et al.* 2003) it was hypothesised that increased resistin levels may be associated with atherosclerosis. This hypothesis

was approached by applying different methods. Associations between resistin gene variants and the prevalence of vascular diseases were studied. The link between the plasma resistin concentration and carotid artery IMT was also tested. In addition, the expression of resistin and its modulators in different leucocytes were examined as well as the effects of resistin on endothelial cells *in vitro* were tested.

6.2.1 RETN SNPs and vascular diseases

In the type 2 diabetic study group (article I), we observed significant association between the SNPs -420C>G and +299G>A with cerebrovascular disease. In the each SNP, subjects with the rarest genotype that tended to have the highest GHb_{A1}, LDL cholesterol, and systolic blood pressure values, had more often cerebrovascular disease than the others (see article I, tables 2–4). However, the association of resistin SNPs with cerebrovascular disease seems to be independent of the common risk factors. The latter conclusion is supported by the notion that when the risk factors of cerebrovascular disease were included into the statistical model as co-factors, the association between resistin SNP and cerebrovascular disease remained significant.

The risk factors of cerebrovascular disease are hypertension, increased triglyceride and reduced HDL cholesterol concentrations, elevated levels of small, dense LDL particles, and impaired endothelial and platelet function, which are all abundant in type 2 diabetes (Beckman *et al.* 2002). In the present study, 13.6% (n = 35) of the subjects with type 2 diabetes suffered from symptoms of cerebrovascular disease. The difference between genotypes was clear, for example the prevalence of cerebrovascular disease was three times higher in the G/G genotype of SNP-420C>G than in the other two genotypes, however, the small number of subjects undermines the reliability of this association. Moreover, the resistin SNPs were not associated with the prevalence of coronary heart disease.

However, support for our finding comes from the study of Efstathiou and colleagues (Efstathiou *et al.* 2007). They reported that the plasma resistin concentration measured from patients within 24 hours from the first-ever atherothrombotic ischemic stroke onset predicted the 5-year post-stroke mortality, which was 80% in the highest resistin tertile compared to 16% in subjects of the lowest tertile (Efstathiou *et al.* 2007). Though the SNP-420C>G has been linked to the higher plasma resistin level (e.g. article II, Osawa *et al.* 2007, Yamauchi *et al.* 2008), we cannot rule out the possibility that association to cerebrovascular

disease is due to some other functional variants in linkage disequilibrium with the SNPs studied or it can represent a false positive result.

The OPERA study population consists of hypertensive and control groups. The hypertensive group can be considered as a high CAD risk population since in both sexes the hypertensive group had significantly higher BMI, blood pressure, blood glucose, plasma insulin, and plasma triglyceride values than the control group (Rantala *et al.* 1998). Genotype frequencies in SNP-420C>G and +157C>T did not differ between these study groups (original article II). However, the genotype frequency of SNP+299G>A as well as the allele frequencies of all three SNPs differed between the hypertensive and control population. The problem in these results is that the common allele and common allele homozygotes were more frequent in the high risk population though most studies have linked the rare allele to a poorer metabolic state (e.g. Norata *et al.* 2007a, Tang *et al.* 2008). In the control population, the common alleles of SNPs -420C>G, +157C>T and +299G>A were associated with lower plasma resistin level. The plasma resistin level has been shown to be higher in young healthy offspring of hypertensive parents compared with healthy offspring of normotensives (Papadopoulos *et al.* 2008). Against this background it is difficult to assess the significance of the difference between OPERA groups.

The discrepancy between the present report and previous studies may be attributable to several factors. First, study populations and study designs differ and risk alleles may vary across different populations. Secondly, interactions with other genes and environmental factors may partly explain the discrepancy in these genetic association studies. (e.g. Gambaro *et al.* 2000, Hegele 2002, Risch 2000.) In addition, the division to the hypertensive and control group was not as clear as it should have been. In the medical examination, some of the control subjects were observed to have high blood pressure previously undetected and untreated. Nonetheless the reliability of the association with SNP-420G>C C allele to a more unfavorable metabolic profile needs to be questioned and tested in further studies.

6.2.2 Plasma resistin level and association to IMT

As described above *RETN* SNPs were associated with the prevalence of cerebrovascular disease (article I). Resistin has also been reported to increase the expression of some adhesion molecules in endothelial cells *in vitro* (Kawanami *et al.* 2004, Verma *et al.* 2003). Since resistin was associated with these factors, we

hypothesized that the plasma resistin concentration could correlate positively with the IMT of carotid arteries (article III). IMT is an intermediate phenotype of early atherosclerosis and it has been shown to predict future vascular events (e.g. in the recent meta-analysis of Lorenz *et al.* 2007). The resistin concentration correlated negatively with mean, ICA, and CCA IMT measurements before adjustments, though the correlation coefficients were weak. After adjustment for other risk factors these correlations became insignificant. Classical risk factors age, sex, smoking, LDL cholesterol and systolic blood pressure were significant explanatory factors for mean IMT in this study population. Therefore, we speculate that resistin does not seem to have a strong independent effect on early atherosclerosis as evaluated via IMT measurements.

Others have reported results differing from our findings. In Japanese subjects with type 2 diabetes, resistin was shown to be positively associated with IMT (n = 155) but this link was not seen in nondiabetic, hypertensive subjects (Takata *et al.* 2008). In another study of Japanese individuals with type 2 diabetes (n = 231), no such link was observed between the plasma resistin level and age corrected mean IMT measurements (Matsuda *et al.* 2004). However, in the same study, a positive association was observed with age corrected maximum IMT and the plasma resistin level (Matsuda *et al.* 2004). In a Caucasian population, the resistin concentration was not detected to associate with IMT when adjusted for age and sex either in subjects with type 2 diabetes (n = 84) or in controls (n = 85) (Dullaart *et al.* 2007). In a larger study of 1090 subjects, the association between resistin and carotid IMT was not detected at baseline and also the correlation between plasma resistin concentration and 2-year IMT progression was not significant (Spearman correlation coefficient 0.06, $P = 0.07$) (Norata *et al.* 2007b). In these reports, it has not been clarified whether the association of resistin with IMT is independent of other risk factors of atherosclerosis, which is a clear limitation. To summarize, the plasma resistin level does not appear to be strongly linked with IMT.

Several studies showed that CAD patients had a higher plasma resistin level than controls (studies are summarized in Tables 3 and 4). Furthermore, these studies demonstrated also that plasma resistin concentrations were higher in the more acute CAD cases such as acute MI and unstable angina pectoris compared to stable CAD cases (Hu *et al.* 2007b, Lubos *et al.* 2007, Pilz *et al.* 2007, Qiao *et al.* 2007). To sum up, the plasma resistin level seems to be elevated in acute CAD cases whereas the association with carotid artery IMT is not clear. In the IMT studies, study populations have not consisted of CAD patients. It is possible that

the association between plasma resistin level and IMT is not apparent in the earlier stages of the disease. The presence of higher plasma resistin in acute CAD cases may be only a sign of ongoing inflammatory reaction associated with infarcted heart tissue.

Follow-up studies for only a few years have presented results from the resistin level in plasma and its prognostic value for future CAD events. Lubos and co-workers have detected an independent effect of the plasma resistin concentration with risk of future CV death (Lubos *et al.* 2007) whereas other smaller studies have not shown this kind of effect (Hoefle *et al.* 2007, Liang *et al.* 2008, Lim *et al.* 2008, Pilz *et al.* 2007). Larger and more extended follow-up studies are needed before the significance of the plasma resistin concentration as a predictive measurement of future CAD can be clarified.

6.2.3 Resistin expression in several white blood cell types

When the association of the plasma resistin level with IMT and the risk factors of atherosclerosis in middle-aged OPERA control group were studied, it was discovered that the number of leucocytes in the blood was the most important explanatory factor of the plasma resistin concentration (original article III). This finding is in accordance with the expression of resistin reported in PBMCs (e.g. Nagaev *et al.* 2006, Xu *et al.* 2007). After this concept was reported, several others have replicated this correlation (Anderson *et al.* 2007, Hu *et al.* 2007b, Konrad *et al.* 2007, Lubos *et al.* 2007).

Resistin correlated also with hsCRP in the OPERA control group independently from obesity markers. HsCRP is a marker of inflammation that is also reported to be a risk factor of atherosclerosis (e.g. Jousilahti *et al.* 2001). Several research groups have described this association between plasma resistin and CRP concentrations (most recently by Aquilante *et al.* 2008, Piestrzeniewicz *et al.* 2008). Based on association studies, it could not be determined whether resistin and CRP were both markers of inflammation or, could they have direct mutual effect on each other. Recently, it has been shown that CRP can stimulate directly the expression of resistin in a dose- and time-dependent manner in PBMCs (Hu *et al.* 2007a).

The blood leucocyte population consists of several different types of cells; neutrophils, eosinophils, basophils, monocytes as well as T and B lymphocytes (see Fig. 2). Of these, the monocytes/macrophages are considered to be the most important in the development of atherosclerosis. However, other leucocytes

involved both in innate and adaptive immune system have been implicated in the atherosclerotic disease process (reviewed by Vanderlaan & Reardon 2005). Resistin has been shown to be expressed in PBMC fraction of blood leucocytes (e.g. Nagaev *et al.* 2006, Xu *et al.* 2007) and in primary monocytes (Osawa *et al.* 2005, Sundén-Cullberg *et al.* 2007). Since the expression of resistin had not been studied more precisely, an experiment was designed to clarify this issue (article IV). Resistin expression (Ct values below 33) could be detected in all the studied primary cells and cell lines except for THP-1 and NK-92 cells. The highest resistin expression was discovered in monocytes and +CD15 positive neutrophils and eosinophils (NE) (see Table 7). Since there are fewer number of eosinophils compared to neutrophils the detected expression of resistin in NE is concluded to be attributable to that produced by neutrophils. Interestingly mouse *Retnlg* has been reported to be expressed in granulocytes as well (Schinke *et al.* 2004).

Šenolt and co-workers have published immunohistochemistry data, that showed co-localisation of resistin with macrophages (CD68), B lymphocytes (CD20), and plasma cells (CD138) but not with T lymphocytes (CD3) in synovial tissue of patients with rheumatoid arthritis (Senolt *et al.* 2007). Their results are consistent with the present study except in the case of T lymphocytes. Immunohistochemistry is not as sensitive a method as RT-PCR and that could be the reason why Šenolt and co-workers did not detect resistin in T cells. The results of the present study can be considered reliable since both relative mRNA and protein expressions of resistin were studied in the cells. However, we cannot rule out the possibility that in certain cases and circumstances such as in different stages of the cell cycle or in diseases the expression of resistin in the studied cells could be different and also below the detection limit. In conclusion, resistin is widely present in leucocyte lineages (Fig. 14).

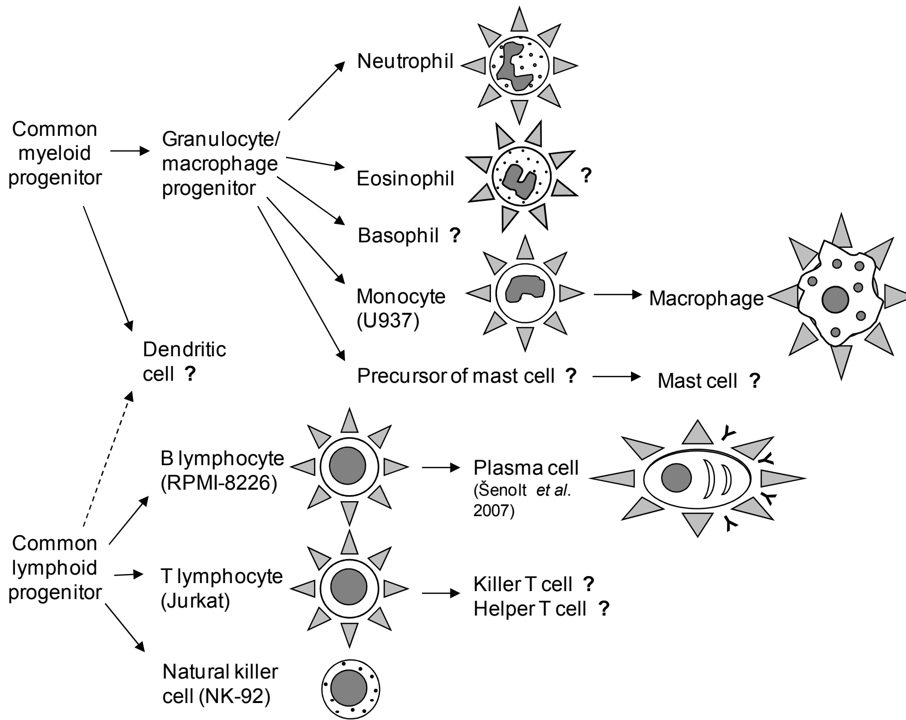

Fig. 14. The expression of resistin in two main leucocyte lineages. Cells presented as suns were showed to express resistin in the present study. Presence of resistin in plasma cells has been shown only by immunohistochemistry (Senolt *et al.* 2007). Question mark describes cell types that have not been studied specifically.

Interestingly, the resistin protein concentration was higher in neutrophils that are the most prevalent white cell population in the blood in comparison with the primary monocytes or the monocytic cell line U937 whereas monocytes had the highest mRNA level. Apparently, resistin protein translation is either regulated differently in neutrophils than in monocytes or resistin is stored after translation inside neutrophils and this accounts for its higher protein level in comparison with the amounts of mRNA. In summary, neutrophils and monocytes seem to be the major source of resistin in the blood.

Neutrophils are often the first cells to come into contact with pathogens. In conjunction with tissue macrophages differentiated from monocytes, neutrophils are the key players of the innate immunity system (Janeway *et al.* 2005). They may help to initiate the immune response and modulate the response by releasing several cytokines (Appelberg 2007, Janeway *et al.* 2005). Both neutrophils and

macrophages produce chemokines that could contribute to the regulation of leucocyte accumulation into the inflammatory site (Osterud & Bjorklid 2003, Scapini *et al.* 2000). Human resistin has also been shown to possess chemotactic properties (Chumakov *et al.* 2004). Therefore it can be speculated that resistin could mediate the inflammatory process by attracting more leucocytes to the infected/inflamed site. However, until more studies have been conducted on this issue only speculation is possible. It does seem likely that since resistin can be produced by several leucocyte populations, it may have an impact on several points of the atherosclerotic process and most probably other inflammatory diseases.

6.2.4 Modulations of resistin expression in different leucocyte lineages

Expression of resistin was high in primary monocytes. In tissues such as in arterial wall, blood monocytes differentiate into macrophages. An increase in the resistin mRNA level has been reported previously in primary PBMC as they differentiated towards macrophages (Lehrke *et al.* 2004, Patel *et al.* 2003, Xu *et al.* 2006) and also in differentiated U937 cells (Xu *et al.* 2006). In the present study also the production of resistin in the monocytic U937 cells during the course of differentiation was studied and it was noticed that mRNA, intracellular and secreted resistin levels all declined after 3 days of macrophage differentiation. A recent study of Nagaev and co-workers described also a decline in the resistin mRNA during 24 hour culture of PBMCs and finally a lower level of expression in differentiated macrophages than in freshly isolated PBMCs (Nagaev *et al.* 2006). Since all the levels of expression in the present study demonstated a similar outcome, the decline in resistin expression during the course of monocyte differentiation would seem to be relevant. Nonetheless, the fact that all of these results were obtained under *in vitro* differentiation conditions has to be kept in mind when conclusions are drawn.

Several studies have shown that LPS and TNFα stimulate expression of resistin in primary PBMC (Bokarewa *et al.* 2005, Kaser *et al.* 2003, Lehrke *et al.* 2004, Lu *et al.* 2002, Sundén-Cullberg *et al.* 2007). It has not been studied previously how these factors influence expression of resistin in other white blood cells. In study IV, it was demonstrated that the expression of resistin in neutrophils as well as in monocytes was increased by LPS and TNFα already after exposure of 2 hours. Surprisingly the resistin expression in T (Jurkat) and B cells

(RPMI-8226) was also modified by TNFα (see summary in Table 7). A similar amount of LPS did not induce any significant changes in these T and B cell lines as compared to that in monocytes and neutrophils. Jurkat and RPMI-8226 cells have been reported to be readily activated by LPS (e.g. Lee *et al.* 2006, Tsaprouni *et al.* 2007) suggesting that our experimental conditions differed from these other workers or the expression of resistin in these leucocyte populations is differently regulated by LPS.

Taken together, these results suggest that resistin expression is up-regulated by compounds like LPS and TNFα which can induce inflammation in the cells of both innate and adaptive immune systems. Since the expression of TNFα, is known to be induced by LPS, thus was not monitored in the present study and therefore, the possibility that some of the effects seen after LPS treatment result from the auto/paracrine production of TNFα cannot be excluded.

6.2.5 Resistin and endothelial changes in atherosclerosis

Resistin has been detected inside the arterial wall (Burnett *et al.* 2005, Jung *et al.* 2006). Resistin produced by different leucocytes inside intima could affect endothelial cells as could the resistin circulating in the blood. In the present study, the effects of resistin on endothelial cells, which could be considered as potential targets for resistin, were examined. A dose dependent increase in monocyte adhesion to endothelial cells after resistin exposure was detected, this probably being attributable to the up-regulation of adhesion molecules MCP-1 and PECAM-1 as well as to the modest increase in EA.hy 926 proliferation. Medium from the resistin transfected COS-7 cells produced also similar but more modest effects.

In a previous study, resistin was not detected to influence monocyte adhesion though it increased VCAM-1 expression in HUVECs (Skilton *et al.* 2005). Others have also shown that human resistin up-regulates VCAM-1 both at the mRNA (Kawanami *et al.* 2004) and the protein level (Kawanami *et al.* 2004, Verma *et al.* 2003). In EA.hy 926 cells, no signs of mRNA expression of VCAM-1 or E-selectin at basal level or after resistin treatment could be detected. However, after stimulation with TNFα, both of these adhesion molecules were detectable. The EA.hy 926 cell line is a hybrid of HUVECs and lung carcinoma cell line A549 (Edgell *et al.* 1983). The discrepancy may be due to the different cells and conditions in these experiments.

The increased expression of MCP-1 in endothelial cells after resistin exposure has been confirmed not only in our studies but also by others (Burnett *et al.* 2005, Takata *et al.* 2008, Verma *et al.* 2003) and in this way differs from the other adhesion molecules. However, the increase in MCP-1 expression was ten times higher after TNFα (10 ng/ml) treatment (data not shown) than that achieved by resistin (50 ng/ml) treatment. The same pattern was evident in the monocyte adhesion assay as well (see Fig. 12). This indicates that resistin may not be as strong an effector in endothelial cells as TNFα.

The results of the present study on endothelial cells support the view that resistin may be involved in the development of atherosclerosis as do studies on SMCs (Calabro *et al.* 2004, Jung *et al.* 2006) as well as monocytes/macrophages (Mu *et al.* 2006, Rae & Graham 2006, Rae *et al.* 2007, Xu *et al.* 2006) (Fig. 15). The plasma resistin level has been shown to be increased in inflammatory conditions such as endotoxemia (Anderson *et al.* 2007, Lehrke *et al.* 2004, Sundén-Cullberg *et al.* 2007) and the present study showed that the mRNA expression and protein secretion is stimulated by LPS and TNFα in leucocytes involved in the innate and adaptive immunity systems. At the same time, resistin itself enhances inflammatory reactions by increasing the production of proinflammatory cytokines in several cell types (Bertolani *et al.* 2006, Bokarewa *et al.* 2005, Kusminski *et al.* 2007, Nagaev *et al.* 2006, Silswal *et al.* 2005). In summary, resistin along with other inflammatory cytokines may create the changes that favour the development of atherosclerosis.

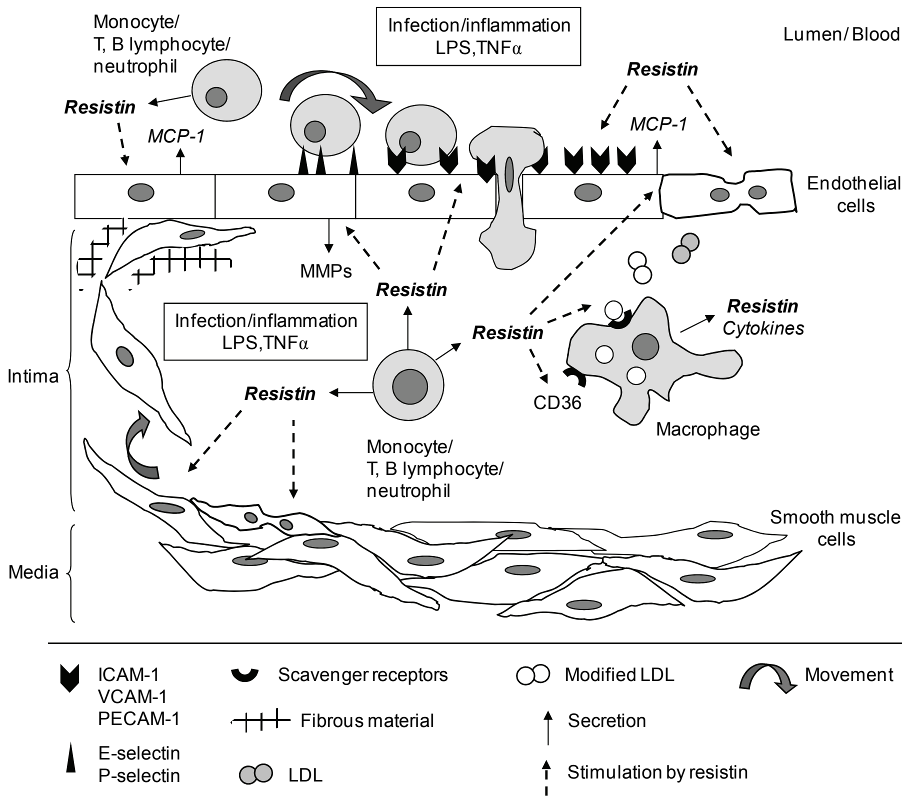

Fig. 15. The effects of resistin in the atherosclerotic lesion. ICAM-1, intercellular adhesion molecule 1; IL, interleukin; LDL, low density lipoprotein; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein 1; MMPs, matrix metalloprotease; PECAM-1, platelet-endothelial cell adhesion molecule 1 TNFα, tumor necrosis factor alpha; VCAM-1, vascular cell adhesion molecule 1.

6.3 Resistin and glucose and insulin metabolism

Initially human resistin was believed to be associated with insulin resistance similarly to murine resistin (Steppan *et al.* 2001a). Therefore, a study comparing *RETN* SNP-420C>G, +157T>C, and +299G>A genotype frequencies between a group of subjects with type 2 diabetes and a control nondiabetic group was deviced. The SNPs in *RETN* were not associated with type 2 diabetes as has been shown by others also for SNP-420C>G in the Finnish population (Conneely *et al.*

2004) and in another Caucasian population (Engert *et al.* 2002, Ma *et al.* 2002) subsamples. The study configuration in the comparison of genotype frequencies between type 2 diabetic and control groups may have influenced the results. The subjects with type 2 diabetes and the healthy controls were not collected at the same time. Subjects in both of these study groups were from the area of Oulu University Hospital. However, because they were not matched for age, BMI etc. this could diminish the possibility to detect significant differences between study groups.

A significant association between the resistin SNPs and mean blood glucose was found in type 2 diabetes study group. SNP-420C>G and +299G>A were also associated with fasting blood glucose and SNP-420C>G with $GHbA_1$ in men. In the subjects with type 2 diabetes, the SNP-420C>G G/G genotype had the highest values in the various glucose variables. However, in the OPERA control population, the C/C genotype had the highest $GHbA_{1c}$ level. The discrepancy between these results and the fact that most of these associations were seen only in one sex leads to the conclusion that these results or at least part of them may be false positive. Moreover, when the plasma resistin levels in the OPERA control population were studied, they were found not to be independently associated with BMI, $GHbA_{1c}$, fasting glucose, or markers of insulin resistance.

In conclusion, the aim of the study was to determine whether the SNPs in *RETN* are associated with type 2 diabetes and its complications. Based on the results and other SNP studies summarized in Table 2, it is concluded that *RETN* and its variations are unlikely to have a strong impact on the development of type 2 diabetes. The results on plasma resistin concentration and its association with type 2 diabetes and insulin resistance have not brought consensus to this matter. The results of the present study on plasma resistin levels in a middle-aged Finnish population show that insulin and insulin resistance are not important factors in the determination of the resistin concentration. Furthermore, the plasma resistin level was not associated with BMI. However, resistin may be associated with obesity and type 2 diabetes related metabolic disturbances via its role in the inflammatory process.

6.4 Methodological aspects and reliability of the results

Plasma samples and their storage

The plasma samples of OPERA used in study II and III were collected about 10 years before the measurement of resistin. The OPERA plasma samples were stored at −20 °C instead of the preferred −70 °C. Since during storage proteins may become partly degraded or aggregated. These changes would affect the concentration of measured protein in immunoassays. It is unknown whether the concentration and structure of resistin is affected by long storage times. The intramolecular structure of resistin is created by disulfide bonds between cysteine residues as well as the intermolecular dimer structure that were reported to be fairly resistant, at least to reducing conditions (Aruna *et al.* 2003). Nonetheless, it is possible that the storage time has changed the resistin in the plasma samples. However, since all the samples were treated similarly, storage per se should not affect the results of the association study.

Over the years, the OPERA plasma samples have been thawed and refreezed a few times. Refreezing cascades could change protein structure and affect antibody recognitition and therefore interfere with the immunoassay results. Other groups have tested the effect of repeated cycles of freezing and thawing on plasma resistin and this procedure does not seem to influence the plasma resistin concentration (Gerber *et al.* 2005, Lee *et al.* 2003).

Genotyping by RFLP

The genotypes of the three *RETN* SNPs were determined by RFLP, since these SNPs altered the recognition sequence of the restriction enzyme. The enzyme cuts the DNA strands if a certain recognition sequence is present. A single nucleotide alteration is enough to prevent the enzyme from recognizing the sequence. It is impossible to know which of the nucleotides in the recognition sequence has been changed and which of the other three possible nucleotides has replaced it. The SNPs used in the present study have been described and sequenced by others (Cao & Hegele 2001, Engert *et al.* 2002, Ma *et al.* 2002, Osawa *et al.* 2002) and the information of the varied nucleotides given in these reports has been used in this study. However, it is not totally certain that in all of the study subjects the changed nucleotides are the same as those described in the original sequencing studies. The efficiency of the restriction enzyme may vary from one experiment to

the next. The success of the restriction reaction was confirmed by evaluating samples from every genotype in every restriction reaction.

Expression studies

RT-PCR is a sensitive method. As reliable results as possible were sought by assaying every sample in duplicate or triplicate in RT-PCR. When samples from the same cell type with different treatments were studied, one reference gene (β-actin or GAPDH) was used. The reliability of the chosen reference was checked at the beginning to rule out any possible effect of the treatment on the expression of the reference gene. When the expression of resistin in different cell types was compared, Ct values of resistin were balanced with the expressions of both reference genes.

Remnants of DNA in the RNA sample are one possible cause for inaccurate results. DNase treatment was not conducted on the RNA samples but the effect of possible DNA contamination in the samples was eliminated by RT-PCR primer planning. Every primer pair was designed to cover parts of two exons linked with an intron. The intron would only be present in the original DNA molecules but not in the complement DNA molecules reverse transcribed from mRNA. A long intron could prevent successful PCR or at least the product would be different in size and it could be detected in by inspection of the RT-PCR melt curve data.

The plasma resistin concentration from OPERA samples was measured in duplicate using the enzyme-linked immunoassay kit (Linco Research Inc., USA) (intra- and interassay coefficients of variation 4.5 and 7.4%, respectively). In our measurements, the interassay coefficient of variation was even lower (5.2%) than that reported by the manufacturer. Protein levels of resistin were measured from cell extracts and cell culture medium samples with an in-house assay in triplicate. In the assays as well as in cell culture experiments, recombinant resistin protein produced in human embryonic kidney cells (HEK 293) was used to guarantee the quality of the protein. Intra- and interassay coefficients of variation in the in-house assay were 3.9 and 8.4%, respectively, which are in the same range as in the commercial assay.

In the validation of the reliability of the in-house resistin assay, the human resistin ELISA from Linco Research was used. The resistin concentrations obtained with the commercial and in-house assay correlated strongly ($r = 0.754$, $p \leq 0.001$) but the concentration quntified with the in-house assay were about 10% higher. The assays are based on different methods with different sensitivities

and also the different antibodies and standards used in the assays can account for the difference in the results. The fact that the results were different between the assays used does not affect the results because they were used in different studies.

6.5 Future aspects

Since resistin expression was previously only reported in monocytes, in this study the main subclasses of leucocytes were explored. Further separation of mast cells, dendritic cells and basophils would provide extended information. Some leucocytes further specialize into different functions i.e. T lymphocytes into killer and helper T cells and the expression of resistin should be examined in these cell types as well.

In the present study, it was discovered that the resistin mRNA level was highest in monocytes whereas neutrophils had the highest intracellular protein concentration of resistin. mRNA stability studies as well as possible storage of the protein could provide answers to this phenomenon. It is still not known how resistin is secreted from the cells. The passage of resistin in different membrane sacs could be monitored for example with fluorescent protein coupling.

In this study it was noted that the expression of resistin decreased along with monocyte differentiation into macrophages. However, *in vivo* macrophages form a very heterogenenous population. They are exposed to mixtures of stimuli and can adopt different functional profiles accordingly. The two main profiles are the "classically activated" M1 macrophages that carry the pro-inflammatory profile and "alternatively activated" M2 macrophages with anti-inflammatory properties. (Reviewed in Van Ginderachter *et al.* 2006.) M1 type macrophages have been shown to express more of the pro-inflammatory marker CD14 and to accumulate more cholesterol spontaneously than M2 macrophages in cell culture studies (Waldo *et al.* 2008). In human coronary arteries, M1 type macrophages have been reported to be predominant in atherosclerotic lesions whereas M2 macrophages dominate in the areas devoid of disease (Waldo *et al.* 2008). Thus it would be interesting to study whether the expression of resistin is altered according to macrophage profile. In view of the effects of resistin summarized in Fig. 3, it could be speculated that resistin would be produced by M1 type of macrophages.

Human population based association studies and cell culture experiments have indicated a certain role for resistin in atherosclerosis. Since murine resistin is differently expressed than human resistin, mouse models of atherosclerosis may not be ideal to study the role of this protein in human. However, porcine resistin is

more homologous with human resistin and expressed also in leucocytes (Dai *et al.* 2006). Pigs are widely used in studies of cardiovascular disease since they display similarities to the human types of disease. Studies on pigs could bring new experimental possibilities to elucidate the role of human resistin.

Here some ideas for future research have been presented which evolved out of the present study. Since resistin has been recognized for less than a decade, many topics still need to be investigated. In the future it is most likely that resistin research will focus more strongly on immunological diseases and related issues.

7 Conclusions

The purpose of this study was to clarify the role of resistin in humans. The aims of each original work and the conclusions are shortly as follows:

I To study the connection between resistin and type 2 diabetes.

- Our study suggests that *RETN* polymorphisms are unlikely to have a strong impact on the development of type 2 diabetes, because allele or genotype frequencies did not differ between type 2 diabetes and nondiabetic control groups.

II To study the connection of *RETN* variants with plasma resistin concentration.

- *RETN* SNPs were associated with the resistin concentration in plasma independently of age, sex, number of blood leucocytes, and BMI. Genetic variation seems to be one of the factors influencing plasma resistin levels.

III To study the association of resistin with atherosclerosis.

- The number of blood leucocytes was the strongest explanatory factor of the variation in plasma resistin levels. The resistin level correlated also with hsCRP. These results imply that inflammatory factors are more important in the determination of the plasma resistin concentration than insulin resistance or BMI. Though the plasma resistin concentration is associated with inflammatory markers it was not independently linked with carotid artery IMT in our middle-aged population.

IV To study the role of resistin in cells related to atherosclerosis.

- Resistin was expressed in all the main leucocyte lineages with monocytes and neutrophils having the highest levels of resistin mRNA and protein. Its expression was up-regulated by LPS and/or TNFα in the cells involved in both innate and adaptive immunity. Therefore resistin could play a role in acute infections as well as in inflammatory diseases. Our results showing that resistin increases adhesion and the expressions of MCP-1 and PECAM-1 support the hypothesis that resistin may have a role in the development of atherosclerosis.

The aims of this study were to obtain answers to some of the questions that have surrounded human resistin. The present studies have indicated that human resistin is more closely linked with inflammatory factors than it is with obesity and insulin resistance. One novel discovery was that resistin is expressed in other leucocytes in addition to monocyte/macrophages, most importantly in neutrophils. These results suggest that resistin is largely involved in inflammatory reactions and at the same time it may influence atherosclerosis in the same way as other inflammatory cytokines. In the future, resistin could possibly be used as a marker of inflammation. To sum up, the studies presented in this thesis have helped to clarify the role of resistin in humans, though several questions still remain to be clarified in the future.

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