OULU 2014
D 1259

ACTA UNIVERSITATIS OULUENSIS

Outi Kummu

HUMORAL IMMUNE RESPONSE TO CARBAMYL-EPITOPES IN ATHEROSCLEROSIS

UNIVERSITY OF OULU GRADUATE SCHOOL;
UNIVERSITY OF OULU,
FACULTY OF MEDICINE,
INSTITUTE OF DIAGNOSTICS,
DEPARTMENT OF MEDICAL MICROBIOLOGY AND IMMUNOLOGY;
MEDICAL RESEARCH CENTER OULU;
NORDLAB OULU;
OULU UNIVERSITY HOSPITAL

ACTA UNIVERSITATIS OULUENSIS
D Medica 1259

OUTI KUMMU

HUMORAL IMMUNE RESPONSE TO CARBAMYL-EPITOPES IN ATHEROSCLEROSIS

Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium F202 of the Department of Pharmacology and Toxicology (Aapistie 5 B), on 14 November 2014, at 12 noon

UNIVERSITY OF OULU, OULU 2014

Acta Univ. Oul. D 1259, 2014

Supervised by
Professor Sohvi Hörkkö
Doctor Chunguang Wang

Reviewed by
Docent Agneta Ekstrand
Professor Johan Frostegård

Opponent
Professor Outi Vaarala

ISBN 978-952-62-0566-3 (Paperback)
ISBN 978-952-62-0567-0 (PDF)

ISSN 0355-3221 (Printed)
ISSN 1796-2234 (Online)

Cover Design
Raimo Ahonen

JUVENES PRINT
TAMPERE 2014

Kummu, Outi, Humoral immune response to carbamyl-epitopes in atherosclerosis.
University of Oulu Graduate School; University of Oulu, Faculty of Medicine, Institute of Diagnostics, Department of Medical Microbiology and Immunology; Medical Research Center Oulu; Nordlab Oulu; Oulu University Hospital
Acta Univ. Oul. D 1259, 2014
University of Oulu, P.O. Box 8000, FI-90014 University of Oulu, Finland

Abstract

Carbamylation of proteins *in vivo* occurs by cyanate, non-enzymatically when urea is dissociated, and by myeloperoxidase-catalyzed oxidation from thiocyanate. Carbamylation of low-density lipoprotein is suggested to enhance atherogenesis in patients with with chronic kidney disease and uremia. This thesis study assessed the questions of whether healthy humans or uremic patients under enhanced carbamylation have antibodies recognizing carbamyl-epitopes in plasma, and what is their role *in vivo*. Also, humoral immune response to carbamyl-LDL immunization and its impact on atherogenesis in $LDLR^{-/-}$ mice was investigated.

In this thesis study, plasma antibodies to carbamylated proteins were detected in humans, and IgG antibodies to carbamylated proteins were associated with uremia and smoking, conditions with enhanced carbamylation. The human IgG and IgM antibodies binding to carbamyl-epitopes were associated with oxidation-specific epitopes in plasma. Monoclonal Fab antibodies with characteristics of a natural antibody and ability to bind both carbamyl- and malondialdehyde-derived epitopes were cloned from healthy humans. An investigated Fab antibody was able to bind epitopes found in atherosclerotic lesions and inhibit the uptake of modified LDL by macrophages. Human plasma antibodies and the monoclonal Fab bound to epitopes found on apoptotic cells. Human B-cells secreted antibodies with similar cross-reactive binding properties between carbamyl- and malondialdehyde adducts and apoptotic cells *in vitro*. Immunization with mouse carbamyl-LDL without adjuvant resulted in specific IgG immune response in $LDLR^{-/-}$ mice, but also a cross-reaction with malondialdehyde-adducts was observed. Carbamyl-LDL immunized mice had enhanced plasma antibody binding to apoptotic cells. Carbamyl-LDL immunization did not affect atherogenesis in mice.

This thesis demonstrates that IgG antibodies to carbamyl-epitope might serve as a novel indicator of carbamylation *in vivo* in uremic patients or smokers. The cross-reactivity between antibodies binding to carbamylated and oxidation-specific epitopes, and apoptotic cells may have a role in explaining the link between enhanced atherogenesis and kidney disease.

Keywords: antibodies, apoptosis, atherosclerosis, carbamylation, cross reactions, epitopes, humoral immunity, low-density lipoprotein, malondialdehyde, uremia

Kummu, Outi, Humoraalinen immuunivaste karbamyyli-epitooppeja kohtaan valtimonkovettumataudissa.

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta, Diagnostiikan laitos, Lääketieteellinen mikrobiologia ja immunologia; Medical Research Center Oulu; Nordlab Oulu; Oulun yliopistollinen sairaala

Acta Univ. Oul. D 1259, 2014

Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä

Proteiinien karbamylaatiota tapahtuu syanaatin vaikutuksesta. Sitä muodostuu urean hajotessa tai myeloperoksidaasin katalysoimana tiosyanaatin hapettuessa. Low-density lipoproteiinin eli LDL:n karbamylaation on esitetty edistävän valtimonkovettumataudin eli ateroskleroosin kehittymistä munuaisten vajaatoimintaa sairastavilla ureemisilla potilailla. Väitöskirjatyössä tutkittiin, onko terveillä ihmisillä ja ureemisilla potilailla karbamyyli-epitooppeja tunnistavia vasta-aineita, ja mikä niiden merkitys on elimistössä. Humoraalista immuunivastetta karbamyyli-LDL-immunisaation jälkeen sekä sen vaikutusta ateroskleroosin kehittymiseen tutkittiin LDL-reseptoripuutteellisilla hiirillä.

Tutkimuksessa osoitettiin, että ihmisillä on plasmassa karbamyloituja proteiineja tunnistavia vasta-aineita. IgG-luokan vasta-aineet ovat yhteydessä uremiaan ja tupakointiin, joissa karbamylaatio on lisääntynyt. Karbamyyli- ja hapettuneita epitooppeja tunnistavien plasman IgG- ja IgM-vasta-aineiden välillä havaittiin olevan yhteys. Työssä kloonattiin terveistä ihmisistä monoklonaalisia Fab-vasta-aineita, joilla on luonnollisten vasta-aineiden kaltaisia ominaisuuksia ja kyky sitoutua sekä karbamyyli- että malonidialdehydi-epitooppeihin. Yksi tutkittu Fab-vasta-aine sitoutui valtimonkovettumataudin ateroomissa oleviin epitooppeihin ja esti muuntuneen LDL:n sisäänoton makrofagi-soluihin. Ihmisen plasman vasta-aineet ja monoklonaalinen Fab-vasta-aine sitoutuivat apoptoottisten solujen pinnalla oleviin rakenteisiin. Soluviljelyolosuhteissa ihmisen B-solut tuottivat vasta-aineita, joilla oli samanlaisia ristireaktio-ominaisuuksia karbamyyli- ja malonidialdehydi-epitooppeja sekä apoptoottisia soluja kohtaan. Karbamyyli-LDL-immunisaatio sai aikaan IgG-immuunivasteen hiirillä karbamyyli-LDL:a kohtaan, mutta myös ristireaktio malonidialdehydi-rakenteita sekä apoptoottisia soluja kohtaan havaittiin. Karbamyyli-LDL-immunisaatio ei vaikuttanut ateroskleroosin kehittymiseen hiirillä.

Tutkimus osoittaa, että IgG-vasta-aineet karbamyyli-epitooppeja kohtaan voivat olla uudenlainen karbamylaation merkkiaine elimistössä ureemisilla potilailla ja tupakoitsijoilla. Karbamyloituneiden ja hapettuneiden epitooppien sekä apoptoottisten solujen välillä havaituilla vasta-aineiden ristireaktioilla voi olla merkitystä valtimonkovettumataudin etenemiseen munuaisten vajaatoiminnassa.

Asiasanat: apoptoosi, ateroskleroosi, epitoopit, karbamylaatio, low-density lipoproteiini, malonidialdehydi, ristireaktiot, uremia, vasta-aineet, vasta-aineiden muodostus

To Dad,
I wish you were here to see me now

Acknowledgements

This study was carried out at the medical campus of University of Oulu. The work was started at the Department of Internal Medicine, University of Oulu and continued at the Clinical Research Center, Oulu University Hospital. The last few years I spent at the Department of Medical Microbiology and Immunology, University of Oulu. I have been offered great facilities for my research during these years, and I am grateful for Professor Antero Kesäniemi and Professor Olli Vainio for giving me the opportunity to work at these departments, and under their guidance.

My deepest, sincere thanks go to my principal supervisor, Professor Sohvi Hörkkö, who has patiently guided me on my journey to become a researcher. Sohvi has taught and supported me, yet giving me a freedom to make mistakes and learn on my own. I will always be grateful to Sohvi for introducing me the fascinating world of antibodies.

I also want to warmly thank my other supervisors. At the beginning of my research career, Professor Antero Kesäniemi gave me an opportunity to work in the field of atherosclerosis research, and to learn several techniques used in the lipid research. During the last few years, Dr. Chunguang Wang shared her interest towards phage display and Fabs with me, and has supported me through the hardest part of my thesis work, publishing the research.

I wish to thank all co-authors and collaborators for their input and expertise. Without your contribution this work might not have been possible. Dr. Helena Kastarinen is thanked for her friendly attitude and help recruiting the patients with chronic kidney disease, and expertise in nephrology. Professor Juha Risteli and Dr. Marja-Kaisa Koivula are acknowledged for collaboration regarding homocitrulline measurements. Dr. Piotr Prus is thanked for expert help with Biacore.

I wish to express my gratitude to all former and present members of Sohvi's research group. Your help and cheerful company have made my work easier. Especially Sirpa Rannikko, Pauliina Turunen, Antti Nissinen, Marja Veneskoski, Jaakko Lehtimäki and Kirsi Harila have contributed greatly to this thesis project. When I started as a young student, I shared an office with Eija Nissilä (née Kellokoski). Even though we had different research interests, we were able to learn and use the same methods and techniques. I want to thank Eija for broadening my view, and for the amazing time we spend together when visiting Sohvi in San Diego. Later I was privileged to get to know Pauliina, who shared

with me all the ups and downs of a PhD student, sitting next to me in the lab and in the office. Pauliina's support and friendship got me through the hardest days, and her great sense of humor, determined attitude and magic touch in the laboratory experiments will always remain in my memories. Antti has always had time for my questions, and usually also the answer. His knowledge and experience, and positive attitude are highly appreciated. Sirpa is the one person, who has always been around when everything else has changed. Her encouragement, friendship and professional help have been indispensable. Without Sirpa I would not have made it.

I also want to warmly thank all staff members in the research laboratory of the Department of Internal Medicine, in the Clinical Research Center and at the Department of Medical Microbiology and Immunology. I have met plenty of skillful and kind people who have helped me during these years. Special thanks to Riikka Syrjänen for all the help and support I was given during the final stage of my thesis.

My thesis pre-examiners, Docent Agneta Ekstrand and Professor Johan Frostegård are acknowledged for their critical review of the thesis manuscript, and their favorable statements. I appreciate the comments and suggestions given to improve my thesis. I would like to thank Matti Timonen for the careful revision of English language, and Saana Piltonen for revising the Finnish abstract.

I want to thank everyone who voluntarily participated in this project by giving blood samples for my studies. Your contribution was crucial for my thesis.

My family, your love and support during my whole life have been valuable. Ville, you have certainly provided a different perspective on many things in life, and helped me keep my feet on the ground. Thanks for pushing me forward, even when I was out of my comfort zone. My dearest, Sami and Laura, you have taught me what is important in life. Thank you.

This study was financially supported by the Academy of Finland, the Finnish Foundation for Cardiovascular Research, the Aarne Koskelo Foundation, the Ida Montin Foundation and the Medical Research Center Oulu.

Oulu, September 2014 Outi Kummu

Abbreviations

apoB	apolipoprotein B
apoB/E	apolipoprotein B/E
apoE$^{-/-}$	apolipoprotein E -deficient
ACPA	anti-citrullinated protein antibodies
BHT	butylated hydroxytoluene
bp	base pair
BSA	bovine serum albumin
C	constant
CAD	coronary artery disease
cDNA	complementary DNA
CDR	complementarity determining region
CuOxLDL	copper oxidized low-density lipoprotein
CKD	chronic kidney disease
CRP	C-reactive protein
CVD	cardiovascular disease
D	diversity
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunoassay
eNOS	endothelial nitric oxide synthase
ESRD	end-stage renal disease
Fab	antigen binding fragment
Fc	crystallizable fragment
GFR	glomerular filtration rate
H	heavy
HAMA	human anti-mouse antibody
HAT	hypoxantine-aminopterin-thymidine
HCit	homocitrulline
HD	hemodialysis
HDL	high-density lipoprotein
HFD	high fat diet
4-HNE	4-hydroxynonenal
HPLC	high-performance liquid chromatography

HUVEC	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule-1
IDL	intermediate-density lipoprotein
Ig	immunoglobulin
IMT	intima-media thickness
i.p.	intraperitoneal
i.v.	intravenous
IPTG	isopropyl-thiogalactosidase
J	joining
kDa	kilodalton
L	light
LB	Luria broth
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LOX-1	lectin-like, oxidized low-density lipoprotein receptor-1
MAA	malondialdehyde acetaldehyde
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MDA	malondialdehyde
MMP	matrix metalloproteinase
MOPS	3-(N-morpholino)propanesulfonic acid
MPO	myeloperoxidase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B-cells
NO	nitric oxide
NZW	New Zealand White
OxLDL	oxidized low-density lipoprotein
PBHCit	protein-bound homocitrulline
PBS	phosphate buffered saline
PC	phosphocholine
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI	propidium iodide
POVPC	1-palmitoyl-2-(5'-oxovaleroyl)-sn-glycero-3-phosphocholine
RAG	recombination activating gene
RLU	relative light unit
RNA	ribonucleic acid
ROS	reactive oxygen species

RSS	recombination signal sequence
SB	Super broth
scFv	single-chain variable fragment
s.c.	subcutaneous
SCN^-	thiocyanate
SD	standard deviation
SLE	systemic lupus erythematosus
SRA	scavenger receptor class A
TBARS	thiobarbituric acid reactive substances
TBS	tris buffered saline
TNBS	2,4,6-trinitrobenzenesulfonic acid
UV	ultraviolet
V	variable
VCAM-1	vascular cell adhesion molecule-1
V(D)J	variable (diversity) joining
V_H	heavy-chain variable region
V_L	light-chain variable region
VLDL	very low-density lipoprotein

List of original publications

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:

I Kummu O, Turunen SP, Wang C, Lehtimäki J, Veneskoski M, Kastarinen H, Koivula MK, Risteli J, Kesäniemi YA & Hörkkö S (2013) Carbamyl adducts on low-density lipoprotein induce IgG response in $LDLR^{-/-}$ mice and bind plasma autoantibodies in humans under enhanced carbamylation. Antioxid Redox Signal 19(10): 1047–1062.

II Kummu O, Turunen SP, Prus P, Lehtimäki J, Veneskoski M, Wang C & Hörkkö S (2014) Human monoclonal Fab and human plasma antibodies to carbamyl-epitopes cross-react with malondialdehyde-adducts. Immunology 141(3): 416–430.

III Kummu O, Turunen SP, Harila K, Nissinen AET, Kastarinen H, Wang C & Hörkkö S (2014) Hemodialysis patients have plasma IgG autoantibodies binding to apoptotic cells. Manuscript.

Contents

1 Introduction

Atherosclerosis is the underlying cause of cardiovascular disease, a major cause of mortality and morbidity worldwide. The most important risk factor for atherosclerosis is elevated plasma low-density lipoprotein concentration. Other important risk factors are e.g. hypertension, obesity, diabetes and smoking (Libby 2002, Libby *et al.* 2011, Payne 2012, Weber & Noels 2011). These traditional risk factors alone fail to explain the increased prevalence of atherosclerosis observed in patients with chronic kidney disease (Bevc *et al.* 2006, Drüeke & Massy 2010, Roxborough & Young 1995).

A key player in the initiation and development of atherosclerosis is modified low-density lipoprotein (LDL). LDL carries cholesterol in the blood, and can be trapped in the arterial intima. LDL undergoes modification, for example oxidation, and becomes atherogenic. Modified LDL is recognized by macrophage scavenger receptors and internalized. Lipid filled macrophages, foam cells, constitute the early atherosclerotic lesions (Glass & Witztum 2001).

Carbamylation is a post-translational modification that occurs mainly on the lysine-residues of proteins and peptides creating carbamyl-lysine, also called homocitrulline. Carbamylation is caused by cyanate, which can be formed *in vivo* by spontaneous degradation of urea, or by myeloperoxidase-catalyzed oxidation of thiocyanate. Non-enzymatic carbamylation is dependent on the plasma urea concentration, and is enhanced in chronic kidney disease patients with increased urea levels. Myeloperoxidase-catalyzed carbamylation has been linked to inflammation and increased thiocyanate levels found e.g. in smokers. Carbamylation of LDL has been suggested to promote atherogenesis in conditions with enhanced carbamylation (Kraus & Kraus 2001, Wang *et al.* 2007).

Carbamylated LDL has several atherogenic properties. It is recognized and taken up by macrophage scavenger receptors and is able to induce endothelial cell dysfunction (Basnakian *et al.* 2010). In addition, carbamyl-LDL is shown to be immunogenic in guinea pigs and rabbits, leading to antibody formation (Apostolov *et al.* 2005, Steinbrecher *et al.* 1984a). Antibody responses to atherogenic lipoproteins, mainly oxidation-specific epitopes on oxidized LDL, have been widely studied, but the role is not unambiguous (Lopes-Virella & Virella 2010). Humoral antibody responses to carbamyl-epitopes have not been studied in detail, and the role of humoral immunity triggered by carbamyl- LDL in atherogenesis is not known.

This thesis study investigated the humoral immune response to carbamyl-epitopes in humans and mice, and its functionality related to atherogenesis *in vivo*.

2 Review of the literature

2.1 Atherosclerosis

Atherosclerosis is a chronic and multifactorial vascular disease affecting the large- and medium-sized arteries. An artery has three layers; intima, media and adventitia. The luminal side of an artery is covered with endothelium that is in contact with the blood. The innermost intima consists of extracellular matrix proteoglycans and collagen, with some embedded smooth muscle cells. Underlying media is separated from intima by the internal elastic lamina and consist of several layers of smooth muscle cells embedded in tightly packed extracellular matrix containing elastin and collagen. The outermost layer, adventitia, is mostly collagen and is surrounded by external elastic lamina. The disease is characterized by the accumulation of atherosclerotic plaque in the intima layer of the artery, and the disease will progress during lifetime, slowly narrowing the lumen of arteries. The rupture of vulnerable plaque may lead to thrombosis causing ischemia; restricted blood flow and deprivation of oxygen. Atherosclerosis is the underlying cause for coronary artery disease (CAD) which is a major cause of mortality and morbidity, especially in Western countries (Falk 2006, Hansson 2005, Libby 2002, Weber & Noels 2011).

Atherosclerosis is affected by several environmental and genetic risk factors. Traditional risk factors for atherosclerosis are hypercholesterolemia or dyslipidemia (low high-density lipoprotein, high low-density lipoprotein, high triglycerides, or combination of these), hypertension, cigarette smoking, male gender, age, and family history. Obesity, physical inactivity, diabetes, glucose intolerance and metabolic syndrome also increase the risk for atherosclerosis. Newer risk factors recognized include homocysteine, lipoprotein(a), apolipoprotein B, apolipoprotein A and inflammation biomarkers, for example sensitive C-reactive protein (CRP) (Bevc *et al.* 2006, Fruchart *et al.* 2004, Payne 2012).

Patients with chronic kidney disease (CKD) have higher prevalence of atherosclerosis and cardiovascular disease (CVD) (Foley *et al.* 1998, Lindner *et al.* 1974). CVD, especially CAD, is the most common cause for mortality and morbidity in patients with CKD. Chronic kidney disease is now regarded as an independent risk factor for CAD, and the risk increases with the severity of the disease, as reviewed by Afsar *et al.* (2014).

2.1.1 Pathogenesis of atherosclerosis

The pathogenesis of atherosclerosis may still not be fully understood, and the accumulative data have changed the prevailing theory of atherogenesis over time. Previously, atherosclerosis was considered as a pure cholesterol storage disease, but current understanding suggests a complex interplay between cells and molecules, and atherosclerosis as an inflammatory disease.

Carl von Rokitansky promoted his thrombogenic theory in the middle of the 19th century, when he described plaque formation in the vascular inner layer, and atheroma with lipid-rich core. He proposed that the deposits were derived primarily from fibrin and other blood elements. This theory was attacked when Rudolf Virchow identified the presence of intimal deposits in the arteries, and also noted the inflammatory nature of atherosclerotic plaques, presence of cells, connective tissue, and vascular degeneration. Virchow also gave the first hint of familial heritance of atherosclerosis, as reviewed by Libby (2012) and Ventura (2000).

The first animal experiment in 1913 by Anitchow and Chalatow, on rabbits fed with high cholesterol diet, gave evidence for cholesterol accumulation in fatty lesions resembling human atheroma (Anitschkow & Chalatow 1913, translation reprinted 1983). This was in accordance with Virchow's earlier findings of cholesterol accumulated in arterial lesions, and gave rise to a hypothesis claiming that atherosclerotic lesion was caused by infiltration of lipids. Response-to-retention hypothesis was further introduced as enhanced subendothelial retention of positively charged lipoproteins within arterial wall and association with negatively charged proteoglygans (Tabas *et al.* 2007, Williams & Tabas 1995, Williams & Tabas 1998, Williams & Tabas 1998).

Response-to-injury hypothesis was introduced by Russell Ross in 1973 and it is based on endothelial injury or dysfunction as an initiative factor for atherogenesis (Ross & Glomset 1973). According to the hypothesis, atherosclerotic lesion is formed as a response to an injury in arterial endothelial cells and results in their desquamation. Several factors, such as chronic hyperlipidemia, chemical factors, uremia, metabolites, infections, immunologic injury or mechanical factors, may lead to alterations in cell-cell or cell-connective tissue interactions and detachment of cells from the artery wall that would lead to exposure of underlying collagen and allow platelets from the circulation to adhere to connective tissue. The infiltration of platelet-derived factors and plasma lipoproteins or hormones at the sites of injury would lead to migration and

proliferation of smooth muscle cells from the underlying media and the proliferation of intimal smooth muscle cells, and also to formation of new connective tissue as well as intracellular and extracellular lipid deposition (Ross *et al.* 1977).

Oxidative modification hypothesis states that native LDL is not atherogenic. It originated in 1979 when Goldstein and Brown studied the metabolism of macrophages in cell culture, and found that in patients with familial hypercholesterolemia with no functional LDL receptors, macrophage-derived foam cells accumulate in the artery wall similar to hypercholesterolemic patients with normal LDL receptors. They suggested that LDL is altered and taken up by some alternative macrophage receptors. Chemically modified acetyl-LDL, even though not found *in vivo*, bound specifically to macrophages, was internalized and accumulated into macrophages turning them into foam cells *in vitro* (Brown & Goldstein 1983). The macrophage receptor was called acetyl-LDL receptor, but later characterized and named scavenger receptor A (Kodama *et al.* 1988). It was also observed that native LDL incubated with cultured endothelial cells overnight was recognized by peritoneal macrophages specifically and with high affinity, and this was called endothelial cell-modified LDL. It was suggested that the endothelium-induced LDL modification was causing the LDL uptake and foam cell formation (Henriksen *et al.* 1981). It was evidenced in the later studies that LDL is oxidized during incubation with endothelial cells (Hessler *et al.* 1983, Steinbrecher *et al.* 1984b).

2.1.2 Development of atherosclerotic plaque

The earliest form of atherosclerotic lesion, fatty streak, can already be found in infants and young children (Napoli *et al.* 1997). Fatty streaks consist of inflammatory cells, monocyte-derived macrophages and T-lymphocytes (Ross 1999). Recruitment of monocytes/macrophages and their uptake of modified LDL are the major events contributing to the formation of fatty streaks. Positively charged low density lipoprotein associate with negatively charged extracellular matrix proteoglycans and have enhanced subendothelial retention within arterial wall (Tabas *et al.* 2007, Williams & Tabas 1995, Williams & Tabas 1998) and it is modified e.g. by oxidation (Glass & Witztum 2001).

Endothelial cells undergo inflammatory activation and express vascular cell adhesion molecule-1 (VCAM-1) that binds leucocytes (Cybulsky & Gimbrone 1991). Endothelial activation is required and involves also intercellular adhesion

molecule-1 (ICAM-1), platelet activating factor, interleukin-8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1) (Randolph 2014). Once leucocytes attach to the endothelial cells, they enter the intima in junctions between endothelial cells. Several chemoattractant proteins, such as MCP-1 and lymphocyte specific chemoattractants, participate in the process (Libby 2002). Inflammatory responses stimulate migration and proliferation of smooth muscle cells that are mixed with inflammatory cells (Ross 1999). Monocytes in the arterial intima are transformed into macrophages that express surface scavenger receptors, such as scavenger receptor class A (SRA) and CD36. Macrophages take up modified LDL through scavenger receptors and become lipid-laden foam cells, characteristic to an early atherosclerotic lesion. Macrophages secrete growth factors and pro-inflammatory cytokines, amplifying the inflammatory responses in the lesion, and produce reactive oxygen species (ROS). Macrophages also produce matrix metalloproteinases (MMPs), degrading extracellular matrix in plaque's fibrous cap, and are thus involved in the progression and complication of atherosclerotic lesions. Eventually macrophages die and form necrotic core of the atherosclerotic lesion (Randolph 2014). T-lymphocytes in the intima encounter and bind antigens, for example oxidized LDL, leading to their activation and cytokine production. Expression of tissue factors, matrix metalloproteinases and pro-inflammatory cytokines will further promote the development of atherosclerotic plaque. T-helper cells can polarize to produce pro-inflammatory cytokines, known as Th1 cells, or those secreting anti-inflammatory cytokines, Th2 cells (Libby 2002). Th1 cells predominate generally in the atheroma. Oxidized LDL is suggested to enhance differentiation of naïve CD4+ T-cells to Th1, thus promoting inflammation and progression (Newton & Benedict 2014).

The progression of atherosclerosis is illustrated in Figure 1. The lesion can remain stable with small lipid pool and thick fibrous cap, or the lipid pool can become larger and the fibrous cap thinner, making the plaque vulnerable. Rupture of vulnerable plaque allows blood to come into contact with tissue factors, and it coagulates leading to thrombus formation. The vessel can be occluded by the thrombus, resulting in myocardial infarction, or it can be reabsorbed by endogenous or therapeutic thrombolysis. Wound healing response may lead to smooth muscle cell proliferation, migration, and extracellular matrix synthesis causing thickening of the fibrous cap and constriction of the lumen. Restricted blood flow can cause ischemia under increased cardiac demand and lead to clinical symptoms, such as angina pectoris. The major risk factor for atherosclerosis is hyperlipidemia, and lipid lowering can reduce lipid content in

the plaque and calm the inflammatory response in the intima. As a result, atherosclerotic plaque becomes more stable with thicker fibrous cap, arterial lumen is preserved, and blood flow is unrestricted (Libby 2002).

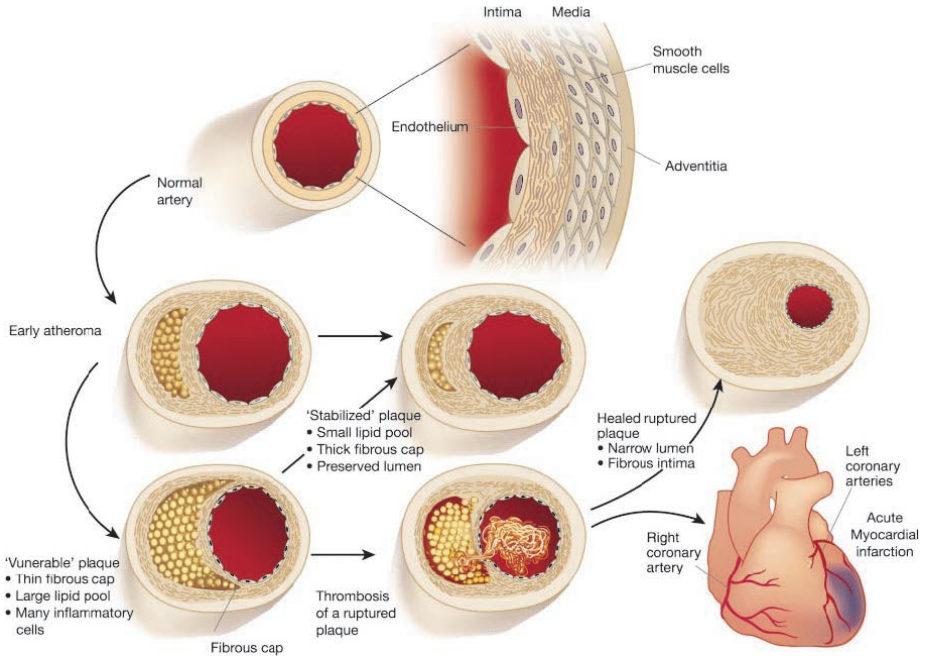

Fig. 1. Development of atherosclerosis (Libby 2002). Reprinted with permission from Nature Publishing Group.

2.2 Carbamylation

Carbamylation is an irreversible, post-translational modification observed in health and disease. Carbamylation occurs spontaneously in the presence of urea in the aqueous environment at physiological temperature and pH. The rate of urea-dependent carbamylation is increased in patients with chronic kidney disease having elevated urea levels. Normal urea level is 5–8 mmol/l but it can be ten times higher in patients with chronic kidney disease. Urea is degraded into ammonium and cyanate, and they form equilibrium in a solution. Cyanate forms reactive isocyanate, which then reacts with amino acids, peptides and proteins, resulting in carbamyl-groups on the molecules (Berlyne 1998, Kraus & Kraus

2001). Cyanate can also be formed when oxidation of thiocyanate is catalysed by myeloperoxidase (MPO). MPO is heme peroxidase found in leukocytes, mostly in neutrophils, but also in macrophages located in atherosclerotic lesions (Sirpal 2009, Wang *et al.* 2007). Thiocyanate (SCN^-) is the preferred substrate for MPO (Wever *et al.* 1982). Normal plasma level of SCN^- varies between 20 and 100 µmol/l in non-smokers and is elevated in smokers (Husgafvel-Pursiainen *et al.* 1987). These two mechanisms of carbamylation are illustrated in Figure 2.

Isocyanic acid is the reactive form of cyanate, and it reacts irreversibly with α-amino and ε-amino groups of amino acids, peptides, or proteins. Carbamylation occurs mainly at α-amino groups on free amino acids. The carbamylation of proteins takes place at ε-amino groups of multiple lysine residues resulting in ε-carbamyl-lysine residues, also known as homocitrulline. Depending on the pH, reversible carbamylation may happen at the hydroxyl-group of tyrosine, serine or threonine, and the sulfhydryl-group of cysteine (Kraus & Kraus 2001, Stark *et al.* 1960, Stark 1964).

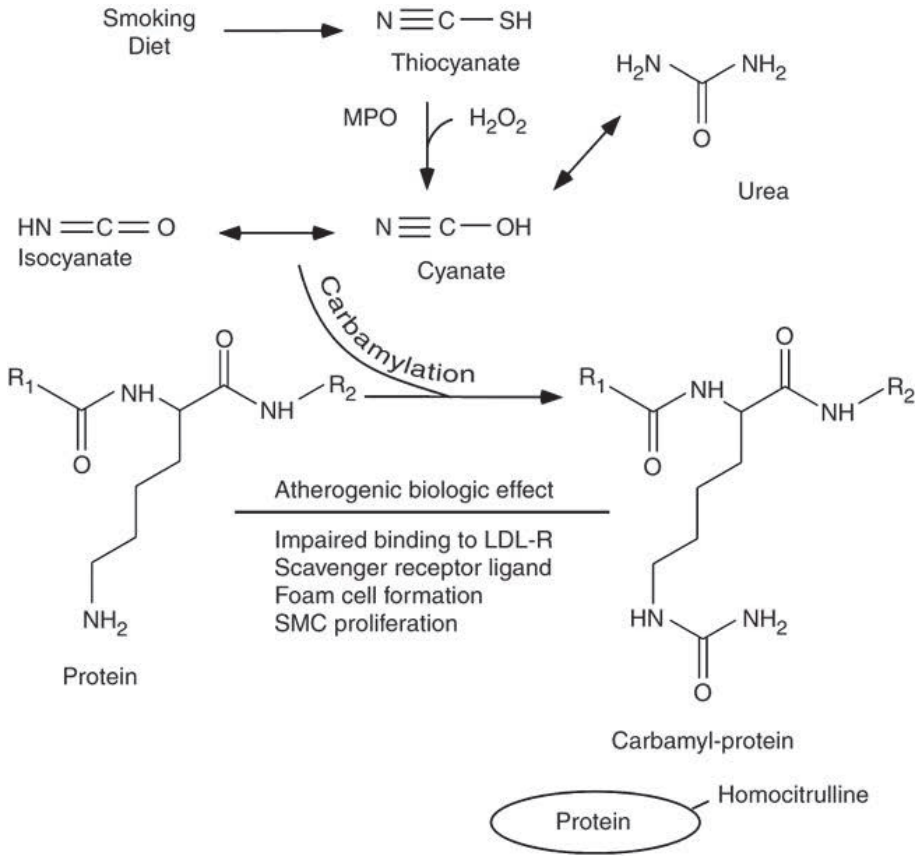

Fig. 2. Carbamylation occurs by cyanate, non-enzymatically when urea is degraded, or by MPO-catalyzed oxidation of thiocyanate (Wang *et al.* 2007). Reprinted with permission from Nature Publishing Group.

Carbamylation can cause structural and functional changes and even loss of function in proteins (Sirpal 2009). Plasma protein carbamylation have been investigated mainly in the context of uremia. Carbamylation related to inflammation, e.g. lipoprotein carbamylation, and relation to cardiovascular diseases has also been of interest (Apostolov *et al.* 2010, Apostolov *et al.* 2012, Hörkkö *et al.* 1992, Hörkkö *et al.* 1994, Ok *et al.* 2005, Wang *et al.* 2007). Recently, carbamylation and anti-carbamyl antibodies have also been studied in patients with rheumatoid arthritis (Shi *et al.* 2011, Shi *et al.* 2013).

2.2.1 Low-density lipoprotein carbamylation

Chemical modification of human LDL by carbamylation neutralizes the positive charge on the ε-amino group of lysine (Weisgraber *et al.* 1978). *In vitro* carbamylation with potassium cyanate does not affect the chemical composition of LDL or its flotation characteristics in ultracentrifugation, but increases the electrophoretic mobility of LDL particles carbamylation time dependently; free amino groups were shown to decrease with increasing carbamylation time (Hörkkö *et al.* 1992).

Cell culture studies have first revealed that LDL binding to its receptor on human fibroblasts is abolished when 15% of lysine residues are carbamylated by cyanate (Weisgraber *et al.* 1978). Also, minimal carbamylation of 1–2% of lysine residues results in a reduced binding of LDL to its receptor. At the same time, modification of 12.5–25% of lysine residues increases the binding of LDL to the macrophage scavenger receptor (Gonen *et al.* 1983). Carbamylation of only 9% of amino groups is shown to decrease the binding to the apolipoprotein B/E (apoB/E) receptor in bovine adrenocortical membranes (Hörkkö *et al.* 1992).

Increased atherogenic properties of carbamylated LDL in patients with CKD on dialysis has already been suggested thirty years ago (Gonen *et al.* 1985), but without a direct and sensitive method carbamylated LDL was not detected in plasma samples of uremic patients. Clearance studies have given evidence for LDL carbamylation *in vivo*. Minor carbamylation (less than 20% or 10 %, respectively) is shown to decrease, but higher carbamylation (over 20% or 14–35%, respectively) is shown to increase the clearance of *in vitro* carbamylated LDL in rabbits (Hörkkö *et al.* 1992) and humans (Hörkkö *et al.* 1994). In addition, uremic LDL clears slower in rabbits compared to LDL isolated from healthy subjects (Hörkkö *et al.* 1994). In humans, LDL clearance is shown to be decreased in uremic patients with conservative treatment, with clearance rate related to the serum urea level (Hörkkö *et al.* 1994), and also in dialysis patients (Hörkkö *et al.* 1995), thus suggesting that LDL carbamylation is contributing to the decreased clearance and accelerated atherogenesis in uremic patients.

Carbamylated LDL, similarly to oxidized LDL (OxLDL) (Galle *et al.* 2001, Heinloth *et al.* 2000), induce dose-dependent vascular cell injuries, including proliferation of vascular smooth muscle cells and endothelial cell death (Ok *et al.* 2005). Cell death is mediated by cell proliferation through the mitogen-activated protein kinase (MAPK) pathway (Apostolov *et al.* 2007). Carbamyl-LDL induced MAPK-dependent mitotic deoxyribonucleic acid (DNA) fragmentation and cell

death has further been shown to be caspase independent and result from increase in endonuclease G expression (Apostolov *et al.* 2011). Carbamylated LDL has also been shown to induce monocyte adhesion to endothelial cells by ICAM-1 and VCAM-1 (Apostolov *et al.* 2007). Carbamyl-LDL exposure also leads to proliferation on human coronary artery vascular smooth muscle cells and increased ICAM-1 and VCAM-1 expression (Asci *et al.* 2008).

Carbamylated LDL binds to lectin-like oxidized LDL receptor-1 (LOX-1), and also to CD36, SREC-1 and SR-A1 receptors on human endothelial cells, and they are essential for the proatherogenic effects of carbamyl-LDL (Apostolov *et al.* 2009). In human umbilical vein endothelial cells (HUVEC) carbamyl-LDL increases reactive oxygen species (ROS) and apoptosis by LOX-1 mediated pathway (Son *et al.* 2011). Carbamylated LDL has been shown to induce endothelial dysfuction, and a recent study suggests a mechanism via activation of LOX-1 receptor leading to p38-dependent NADPH-oxidase activation, production of ROS, endothelial nitric oxide synthase (eNOS) uncoupling and reduced nitric oxide (NO) (Speer *et al.* 2014).

Carbamylation of LDL by uremic serum induces oxidative stress and accelerated senescence in human endothelial progenitor cells similarly to oxidized LDL (OxLDL) (Carracedo *et al.* 2011). Carbamylated LDL and OxLDL share similar proatherogenic properties, such as cytotoxicity and ability to induce cell proliferation and macrophage foam cell formation, and there is a potential competition between these modifications. Carbamylated-oxidized-LDL is shown to be highly cytotoxic to endothelial cells and induce their proliferation. Foam cell formation in mouse primary peritoneal macrophages results from uptake of carbamyl-LDL, OxLDL or carbamyl-oxidized-LDL, by CD36 scavenger receptors, suggesting these two modifications can exist in the same LDL particle (Apostolov *et al.* 2013).

In addition to LDL, carbamylation has also been shown to occur in high-density lipoprotein (HDL) where it is a major post-translational modification. Only one carbamyl-lysine residue in apolipoprotein A-I in HDL is able to induce cholesterol accumulation in macrophages through scavenger receptor class B type I (Holzer *et al.* 2011). It has been suggested that cyanate, rather than carbamylated lipoproteins, induces vascular ICAM-1 expression *in vivo*. Cyanate is able to induce ICAM-1 expression which enhances the neutrophil adhesion in human coronary artery endothelial cells by MAPK pathway p38 and nuclear factor κB (NF-κB). When cyanate is administered in drinking water to mice, it induces ICAM-1 expression in the aorta. Plasma soluble ICAM-1 level also

correlates with plasma protein carbamylation in patients with end-stage renal disease (ESRD) (El-Gamal *et al.* 2012).

2.2.2 Detection of carbamylated proteins

Carbamylated hemoglobin and carbamylated proteins as markers of uremia or indicators of uremic control have been investigated in patients with CKD, as reviewed by Berlyne (1998). Red blood cell survival is diminished in hemodialysis patients (Vos *et al.* 2011), and the prevalence of anemia increases with decreasing kidney function (McClellan *et al.* 2004). Dialysis patients also have hypoalbuminemia caused partly by inflammation and expanded plasma volume, and the increase in plasma volume enhances albumin synthesis (Kaysen & Don 2003, Prinsen & de Sain-van der Velden 2004) which may affect the amount of carbamylated proteins present in plasma. Both long-lived carbamylated hemoglobin and short-lived carbamylated plasma protein concentrations are found to be higher in hemodialyzed patients than in controls. Carbamylated hemoglobin and carbamylated plasma proteins are positively associated and reflect the urea exposure in blood (Balion *et al.* 1998). Total plasma protein carbamylation and plasma carbamyl-LDL have been measured and found to be higher in patients with advanced renal disease on hemodialysis than in healthy controls (Ok *et al.* 2005).

Sandwich enzyme-linked immunoassay (ELISA) method utilizing polyclonal rabbit antibody to carbamyl-LDL has been described to quantify the amount of carbamyl-LDL in human serum samples, and levels of carbamylated LDL is shown to be higher in patients with ESRD compared to controls (Apostolov *et al.* 2005). Carbamyl-modified lysine, homocitrulline, can be detected with high-performance liquid chromatography (HPLC) in protein samples (Turunen *et al.* 2010). Tandem mass spectrometry analysis has been used to detect carbamylated proteins in plasma samples (Berg *et al.* 2013, El-Gamal *et al.* 2012, Jaisson *et al.* 2012, Koeth *et al.* 2013, Wang *et al.* 2007). Protein-bound homocitrulline has shown to independently predict the increased risk for coronary artery disease and major adverse cardiac event (Wang *et al.* 2007). Recently, two separate studies have shown that plasma protein carbamylation is associated with increased mortality in patients with end-stage renal disease (Berg *et al.* 2013, Koeth *et al.* 2013).

Carbamylated proteins have been demonstrated in human atherosclerotic lesions and shown to colocalize with macrophages (Wang *et al.* 2007). Carbamyl-

LDL is detected in mouse atherosclerotic lesions both in proximity of macrophages, but also in macrophage-free zones of intima and plaques (Apostolov *et al.* 2010).

2.2.3 Immunogenicity of carbamyl-LDL and carbamylated proteins

Subtle modification in LDL particle turns it immunogenic, which has been observed in a study with diabetic patients. Nonenzymatic glycation of autologous LDL leads to its rapid clearance from plasma after intravenous (i.v.) injection, and has been shown to result from immune recognition of modified LDL (Witztum *et al.* 1984). Several small modifications, such as glycosylation, methylation, ethylation, acetylation and carbamylation, are shown to cause immunogenicity in homologous LDL and lead to antibody formation in guinea pigs. The antibodies, formed after immunization with modified LDL, are specific for the derivatized lysine residues, and recognized also other similarly modified proteins. Similar cross-reaction is not observed when modified albumin has been used for immunization (Steinbrecher *et al.* 1984a). The study by Steinbrecher *et al.* (1984a) is the first that has demonstrated immunogenicity of carbamyl-LDL *in vivo*. The immunizations have been carried out with and without adjuvant using homologous guinea pig carbamyl-LDL or carbamyl-albumin, and even without adjuvant high-titer antisera are obtained. Polyclonal antibodies against human carbamyl-LDL have been induced in rabbits, but Freund's adjuvant was used in immunizations (Apostolov *et al.* 2005).

Human autoantibodies to carbamylated proteins have very recently been published for the first time in patients with rheumatoid arthritis and arthralgia. The finding of anti-carbamyl-antibodies has come from anti-citrulline-antibodies that are used for prognosis and diagnosis in rheumatoid arthritis. Citrulline and homocitrulline have similar structures, but homocitrulline is one methylene group (CH_2) longer. Anti-carbamylated protein antibodies associates with higher risk for developing rheumatoid arthritis in patients with arthralgia, and more severe disease in patients with rheumatoid arthritis. Anti-carbamyl-antibodies have also been detected in patients that tested negative for anti-citrulline-antibodies, demonstrating to be a distinct serological marker in these patients (Shi *et al.* 2011, Shi *et al.* 2013). Furthermore, some patients with juvenile idiopathic arthritis have shown to have anti-carbamyl-antibodies (Muller *et al.* 2013).

2.3 Oxidized LDL and oxidation-specific epitopes

Lipoproteins are responsible for cholesterol transport in blood. Low-density lipoprotein carries cholesterol from liver to peripheral tissues by LDL-receptor mediated pathway. LDL has molecular mass of 514 kilodaltons (kDa), and it contains one apolipoprotein B 100 (apoB-100) protein. The particle has hydrophobic core consisting mainly of cholesteryl esters and a small amount of triglycerides and unesterified cholesterol, and hydrophilic surface composed of phospholipid layer, free cholesterol and embedded apoB-100 protein (Hevonoja *et al.* 2000). LDL undergoes oxidative modification before it is capable of inducing foam cell formation constituting atherosclerotic fatty streaks. Oxidation of LDL generates a vast variety of conjugates formed by oxidized fatty acids and apoB. Epitopes found on oxidized LDL include phosphorylcholine (PC), malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (Hörkkö *et al.* 2000).

2.3.1 Antibody response to oxidation-specific epitopes

Very small changes in LDL-particle are capable of altering it immunogenic, inducing humoral antibody response (Steinbrecher *et al.* 1984a, Witztum *et al.* 1983, Witztum *et al.* 1984). Antibodies recognize the modified LDL, but often also the modification itself. In addition to small modifications, e.g. glycation, carbamylation, acetylation, ethylation or methylation, breakdown products of oxidatively modified polyunsaturated fatty acids can also modify LDL particles. These reactive breakdown products, such as MDA and 4-HNE, form covalent adducts with lysine residues of apoB and have been shown to induce antibody production in guinea pigs and mice (Palinski *et al.* 1989, Palinski *et al.* 1990). These oxidation-derived epitopes are recognized by the antibodies on oxidized LDL but also in other proteins modified similarly. To emphasize that the epitopes generated during lipid peroxidation could also exist on other structures *in vivo*, they have been named “oxidation-specific”. The oxidation-specific epitopes can be present on proteins, and also on amino-containing headgroups of phospholipids. The decomposed fatty acid in residual phospholipid backbone has reactive aldehyde groups which can form adduct with apoB or lipid moiety of LDL (Hörkkö *et al.* 2000).

Oxidation of LDL and the presence of oxidation-specific epitopes *in vivo* have been evidenced with several findings. The antisera and monoclonal antibodies generated against model epitopes of oxidized LDL, MDA and 4-HNE,

recognize epitopes in atherosclerotic lesions (Palinski *et al.* 1989, Palinski *et al.* 1994, Rosenfeld *et al.* 1990). In addition, LDL extracted from atherosclerotic lesions have shown to contain oxidation-specific epitopes (Palinski *et al.* 1989, Ylä-Herttuala *et al.* 1989, Ylä-Herttuala *et al.* 1994). Autoantibodies to oxidation-specific epitopes are found *in vivo*. Autoantibodies to MDA-LDL have first been demonstrated in rabbit and human sera (Palinski *et al.* 1989) and later in apolipoprotein E -deficient ($ApoE^{-/-}$) mice against oxidation-specific MDA-lysine (Palinski *et al.* 1994) and oxidized LDL (Palinski *et al.* 1996). Autoantibody titers to oxidation-specific epitopes increase with the progression of atherosclerosis in animal models of atherosclerosis. High antibody levels have been observed in cholesterol-fed $ApoE^{-/-}$ mice (Palinski *et al.* 1994), and antibody titers have been shown to correlate with the atherosclerosis in low-density lipoprotein receptor -deficient ($LDLR^{-/-}$) mice after high fat diet (Palinski *et al.* 1995).

2.3.2 Role of antibodies to oxidation-specific epitopes

Natural immunoglobulin M (IgM) antibodies have been cloned against epitopes of copper-oxidized LDL, PC and MDA-adducts from non-immunized $ApoE^{-/-}$ mice demonstrating high antibody titers against oxidation-specific epitopes (Palinski *et al.* 1996). These antibodies recognize epitopes found in atherosclerotic lesions (Palinski *et al.* 1996). These antibodies, e.g. clone named EO6 against PC of oxidized phospholipids, bind to lipid and protein moiety of oxidized LDL, but not to those from native LDL. They also inhibit the uptake of OxLDL by macrophages (Hörkkö *et al.* 1999) and bind to apoptotic cells and inhibit their phagocytosis (Chang *et al.* 1999). Binding of the antibodies to apoptotic cells have been shown to result from recognition oxidation-specific epitopes, such as PC (Chang *et al.* 1999). When apoptotic cells containing oxidation-specific epitopes were used for immunization of mice, they were shown to induce antibody response to oxidation-specific epitopes including PC (Chang *et al.* 2004).

Immunization with oxidized LDL has been shown to confer atheroprotection in animals in several studies (Ameli *et al.* 1996, Freigang *et al.* 1998, Nilsson *et al.* 1997, Palinski *et al.* 1995, Zhou *et al.* 2001). PC is the major epitope on oxidized LDL, and also present in cell wall of gram positive bacteria, e.g. *Streptococcus pneumoniae*. EO6 monoclonal antibody is shown to be genetically identical with previously characterized T15 IgA antibody that is specific for phosphorylcholine conferring protection against *Streptococcus pneumoniae*

(Shaw *et al.* 2000). Pneumococcal vaccination induces high antibody titers to OxLDL demonstrating molecular mimicry between *Streptococcus pneumoniae* and PC, and conferring atheroprotection in $LDLR^{-/-}$ mice (Binder *et al.* 2003).

Autoantibodies to oxidized LDL have been investigated in humans, but their role in atherogenesis is not fully understood. IgG antibodies first described in humans were shown to correlate with the progression of atherosclerosis (Salonen *et al.* 1992). Even though animal studies suggest that immunization with oxidized LDL have a beneficial role, the data from human antibodies to OxLDL have been diverging, as reviewed by Hulthe (2004) and Lopes-Virella & Virella (2010).

Recent view has been that B-1 cell activation and natural IgM production are associated with atheroprotection (Kyaw *et al.* 2011). It has been suggested that IgG antibodies to oxidation-specific epitopes might associate with atherosclerosis and cardiovascular disease, whereas IgM might be protective, thus controversial data exists (Hulthe 2004, Lopes-Virella & Virella 2010). IgM antibody levels to MDA-LDL show inverse association with carotid artery intima-media thickness (IMT) in large cohort study, whereas IgG do not associate (Karvonen *et al.* 2003). Negative association between anti-PC-IgM and development of atherosclerosis in hypertensive patients is described by Su *et al.* (2006). Similarly, IgM levels to OxLDL have negative association to coronary artery disease, and IgG antibodies to OxLDL associated positively to CAD. Despite the associations, IgG or IgM levels were not independent predictors of CAD or clinical events (Tsimikas *et al.* 2007). Low IgM levels to PC, on the other hand, have shown to predict risk of death in hemodialysis patients (Carrero *et al.* 2009). Recently, a 15-year follow-up study revealed that IgG antibodies to copper-oxidized LDL (CuOxLDL) associate with higher risk and IgM antibodies to MDA-LDL associate with lower risk for cardiovascular risk, suggesting that oxidation-specific epitope biomarkers can predict CVD and stroke outcomes (Tsimikas *et al.* 2012).

2.4 Humoral immune response

Immune system consists of innate and adaptive immunity. Innate immunity offers the first-line defense against pathogens; it includes epithelial barrier to prevent infections and phagocytes, natural killer cells, and complement system to eliminate microbes. The innate immune responses occur within hours of infection. Adaptive immunity is mediated by B- and T-lymphocytes and occurs with a delay after the infection. T-lymphocytes participate in the cell-mediated immunity by activating phagocytes to destroy microbes or by killing infected cells, whereas B-

lymphocytes produce antibodies that block and eliminate infections. The immunity mediated by B-cells and antibodies is known as humoral immune response. Antibodies, also called immunoglobulins, are glycoproteins secreted into the body fluids by B-lymphocytes. Membrane-bound immunoglobulins can act as receptors on the surface of B-cells. Secreted antibodies act outside the host cell by binding specifically to a certain structure, or epitopes, on microbes or microbial toxins, and thus neutralizing or eliminating them. Antibodies are able to recognize a vast variety of molecules, including proteins, carbohydrates, nucleic acids, and lipids (Abbas & Lichtman 2005, Abbas *et al.* 2014).

2.4.1 Antibody structure

An antibody consists of two identical heavy (H) chains and two light (L) chains. Each light chain attaches to a heavy chain, and heavy chains attach to each other by disulfide bonds. There are two types of light chains (κ and λ) and five different heavy chains (μ, δ, γ, ε and α). Antibodies are classified into five isotypes in mammals, IgM, IgD, IgG, IgE and IgA, depending on their heavy chains. Antibody light chains have one variable (V) region and one constant (C) region, and heavy chains have one variable and three or four constant regions. Each of the V and C domains is composed of approximately 110–130 amino acids. A hinge region is included between C_H1 and C_H2 regions in antibodies with three C domains. Variable regions are responsible for antigen binding, whereas constant regions constitute the basic framework of antibody. Variable domains of both antibody chains (V_L and V_H) have three hypervariable regions called complementarity determining regions (CDR). These CDR regions are flanked by four less variable framework regions. IgG can be digested with papain into two identical antigen binding (Fab) fragments, both able to bind antigen, and one crystallizable (Fc) fragment. Pepsin digestion results in Fc fragment and single $F(ab)_2$ fragment with ability to cross-link and bind antigen. Monovalent Fv fragments containing only variable domains V_H and V_L, or single-chain variable fragment (scFv) with an additional linker can be genetically engineered (Abbas *et al.* 2014, Schroeder & Cavacini 2010). Antibody structure is shown in Figure 3.

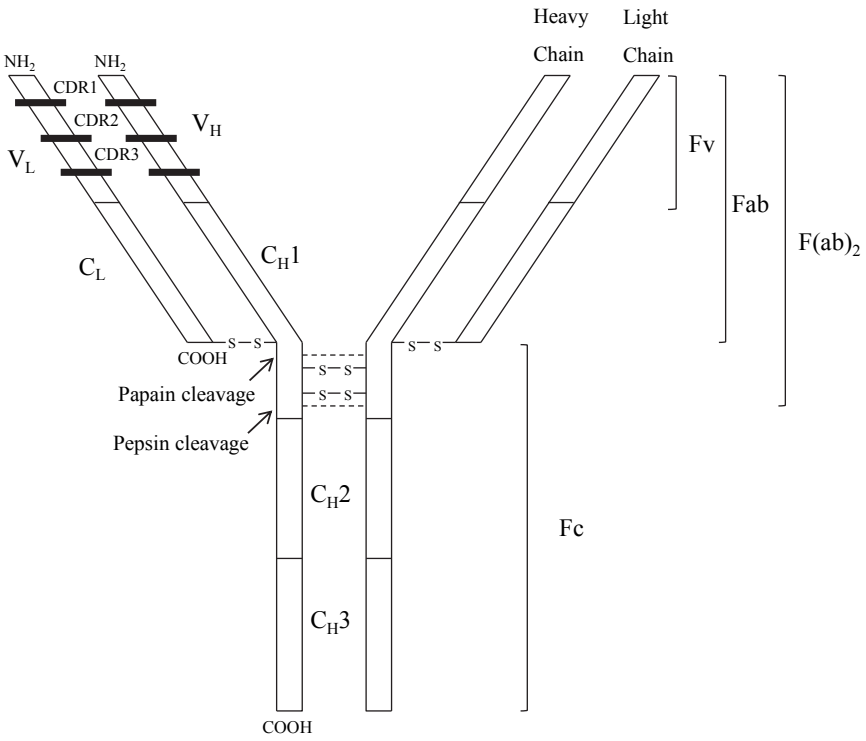

Fig. 3. Illustration of IgG antibody structure (see text in chapter 2.4.1).

2.4.2 Antibody specificity and diversity

Both heavy and light chains contribute to the specificity and diversity of antibodies. Complementarity determining regions are short stretches, about 10 amino acids long, in the antibody variable domains that have the most variability. There are three CDR regions in V_L and three in V_H, and they are named CDR1, CDR2 and CDR3, from amino-terminus of the immunoglobulin chain, of which the CDR3 region is the most variable in both heavy and light chains. All six CDR regions come together when the antibody is correctly folded, creating a three-dimensional specific binding pocket for the antigen. The conserved framework regions adjacent to the CDR region allow the antibody variable region to fold into

the same basic immunoglobulin structure, and still maintain the variability via CDR regions (Abbas & Lichtman 2005, Abbas *et al.* 2014).

The diversity of antibodies, or T-cell receptors, is generated by an assembly of immunoglobulin from germline genes encoding variable (V), diversity (D) and joining (J) gene segments during the maturation of B-cells in bone marrow or T-cells in thymus. Each heavy chain variable region is constructed from V, D and J segments and each light chain from V and J segments. The random combination of gene segments in V(D)J recombination enables the generation of a vast variety of different immunoglobulins, and T-cell receptors with different binding specifities (Abbas *et al.* 2014, Schroeder & Cavacini 2010, Tonegawa 1983).

Heavy and light chains of immunoglobulin are encoded by separate multigene families (Seidman *et al.* 1978, Seidman *et al.* 1980, Tonegawa 1983). Individual V and C regions are encoded independently; the variable V domain is assembled from V(D)J gene segments and the conserved C domains from individual exons. In addition to the coding exons and noncoding introns, each V gene segment contains a recombination signal sequence (RSS) that has conserved heptamer and nonamer sequences separated by 12- or 23- base pair (bp) spacer. This conserved region is exploited in the initiation of the recombination process. Recombination-activating genes (RAGs) 1 and 2 expressed in maturating lymphocytes are required for the initiation of V(D)J recombination, and they act by introducing breaks between rearranging gene segment and its RSS. The breaks are repaired by non-homologous end-joining DNA repair process (Dudley *et al.* 2005).

Combinatorial V(D)J recombination maximizes the diversity encoded by germline genes, but most variation of pre-immune antibody repertoire arises from the junctional diversity in VDJ joining. V_H gene segments can be rearranged by inversion or deletion, and they can be spliced and translated into three different reading frames. In the rearrangement process, germ-line encoded nucleotides can be added, and loss of nucleotides may also occur. Additionally, lymphocytes express terminal deoxynucleotidyl transferase (TdT) which can randomly add nucleotides not encoded by germline genes to the recombination product. V(D)J recombination, CDR3 somatic variation, and pairing of different heavy and light chains all together can yield more than 10^{16} different pre-immune antibodies (Schroeder & Cavacini 2010). Somatic hypermutation is the final mechanism of creating antibody diversity after antigen exposure in variable domain genes, with two mechanisms involved. Mutations are targeted to hot spots with RGYW motif

(Dorner *et al.* 1998) and error-prone DNA synthesis leads to nucleotide mismatch (Rada *et al.* 1998).

Generation of immunoglobulin diversity has hierarchy, starting from D_H / J_H and V_H / DJ_H and later V_L / J_L in B-cell development. Complete and successful light chain production allows expression of IgM on the surface of immature B-cell. C_H genes can undergo alternative splicing, producing either membrane bound immunoglobulin on B-cell surface, or secreted antibody. All immunoglobulins have at first a Cμ heavy chain that can be replaced by another heavy chain constant region in class-switch recombination after appropriate cytokine activation. This allows the VDJ heavy chain variable domain to be attached to any of the heavy chain isotypes (Honjo 1983). IgM and IgD can be simultaneously produced due to the alternative splicing. The B-cells move from bone marrow to the secondary lymphoid organs, constituting the majority of B-cells in spleen. The destiny of B-cell depends on antigen selection. Unstimulated B-cells can survive only days or weeks, but reaction to antigen leads to activation. T-cell independent stimulation results in short-lived plasma cells having limited class-switching properties, whereas T-cell dependent stimulation of B-cell leads to somatic hypermutation of V domains and affinity maturation, unlimited class-switching and generation of long-lived plasma or memory cells (Matthias & Rolink 2005, Schroeder & Cavacini 2010).

2.5 Cloning of monoclonal antibodies

The need for monoclonal antibodies for research, and also for diagnostic and therapeutic purposes, has driven the development of new strategies for antibody cloning. Fully human antibodies are often required for therapeutic use to avoid the immune response against foreign molecules *in vivo* (Nelson *et al.* 2010, Osbourn *et al.* 2003, Yamashita *et al.* 2007).

2.5.1 Hybridoma technique

Hybridoma technique is the first method described for the generation of monoclonal antibodies (Köhler & Milstein 1975). Hybridoma technique is based on the fusion of antibody-producing B-lymphocytes and myeloma cells that can grow indefinitely. Fused hybridoma cells are immortal and secrete antibodies specified by the original B-cell. The method is well established for production of mouse monoclonal antibodies. The lack of suitable fusion partner has hampered

the cloning of human monoclonal antibodies using the hybridoma method, even though some human hybridomas have been cloned (Karpas *et al.* 2001, Karpas *et al.* 2005, Olsson & Kaplan 1980, Vaisbourd *et al.* 2001). Human-mouse hybrids have also been cloned, originating from human B-cells fused with mouse myeloma cell line (Levy & Dilley 1978, Levy *et al.* 1978).

Mouse monoclonal antibodies are usually generated from immunized animals. Mice are immunized with the antigen of interest several times until antibody titers in plasma towards the antigen of interest are observed. Antigens can be mixed with adjuvants to boost the immune response. Several adjuvants are available, of which the commonly used Freund's adjuvant contains emulsified mineral oil and also inactivated *Mycobacterium tuberculosis* in the complete form of adjuvant. Natural antibodies can also be cloned from non-immunized mice. Once antibody response is established, the animal is sacrificed and single-cell suspension is made from the spleen containing antibody-producing B-lymphocytes. Splenocytes are fused with myeloma cells grown *in vitro* with polyethylene glycol (PEG). Cells are grown in the selective media containing hypoxanthine, aminopterin and thymidine (HAT) (Szybalska & Szybalski 1962), which allows only fused hybridomas to grow (Bigda & Koszalka 2013). Unfused splenocytes are able to grow *in vitro* only for some days, and unfused myeloma cells are not able to grow in the presence of aminopterin. Aminopterin blocks the *de novo* synthesis of nucleotides, while hypoxanthine and thymidine can be used as a raw material for alternative nucleotide synthesis. Myeloma cells lack the enzyme for alternative nucleotide synthesis by salvage pathway. Hybridoma-cells surviving the HAT-selection are then tested for the antibody production against the antigens of interest. The goal is to obtain monoclonal cell line originating from one single hybridoma cell, and producing one monoclonal antibody. These monoclonal cell lines can be grown *in vitro* and the produced antibody can easily be purified from the culture media.

2.5.2 Epstein-Barr virus transformation

Human antibody-secreting B-cells can be immortalized by Epstein-Barr virus (EBV) infection (Rosen *et al.* 1977, Steinitz *et al.* 1977). The whole human immunoglobulin molecules that were also present *in vivo* can be produced with this method *in vitro*. The disadvantages of the EBV-transformation are difficult cloning due to the low efficiency of transformation, and the low amount of antibody secreted. Even though the cells can be grown *in vitro*, they cannot divide

indefinitely. EBV-transformed B-cells have shown to have shortened telomers and thus a limited life span (Sugimoto *et al.* 1999, Toda & Sugimoto 2003).

EBV-hybridoma technology combines the traditional hybridoma technique and EBV-transformation of B-cells (Roder *et al.* 1986). The antibody-producing B-cells with desired antigen-specificity are first expanded *in vitro* by EBV-transformation and then fused with suitable cell line. A major problem is the lack of fusion cell lines that would allow stable production of the human antibody (Ainai *et al.* 2006).

2.5.3 Chimeric and humanized antibodies

Mouse monoclonal antibodies with defined specificity are easy to clone using hybridoma technique, but as foreign molecules they will induce human anti-mouse antibody (HAMA) immune response when administered into humans. Different methods have been used to overcome the antigenicity of mouse monoclonal antibodies in humans when applied for therapeutic use. Chimeric mouse–human antibodies have been constructed by combining the variable region genes from mouse antibody with known antigen-binding specificity with the human immunoglobulin constant region genes using recombinant DNA techniques (Morrison *et al.* 1984). The chimeric antibodies are also shown to be immunogenic in mice (Bruggemann *et al.* 1989). One step further in the humanization of rodent antibodies, mouse antibody heavy chain hypervariable regions have been inserted into a human heavy chain combined with a mouse light chain (Jones *et al.* 1986). A breakthrough in the humanization of rodent antibodies has been the insertion of the six hypervariable CDR regions from rat heavy- and light-chain variable domains into human IgG scaffold to generate an antibody with rat antibody specificity against human lymphocytes for therapeutic use (Riechmann *et al.* 1988).

2.5.4 Phage display

Phage display technique was first introduced in 1985 (Smith 1985) for peptide display (Scott & Smith 1990). The technique was soon also applied to antibody fragments (McCafferty *et al.* 1990) and rapidly gained attention as a tool to clone fully human monoclonal antibodies (Barbas *et al.* 1991, Clackson *et al.* 1991, Hoogenboom *et al.* 1991, Marks *et al.* 1991). In 1991 Barbas laboratory published the original pComb3 phagemid system for generation of combinatorial

Fab antibody library (Barbas *et al.* 1991), which has since evolved into pComb3H and pComb3X phagemid vectors (Barbas *et al.* 2001).

The phage display technique allows the generation of fully human antibody fragments, utilizing filamentous phage for assembly of antibody fragments, and disulphide bond formation between heavy and light chains in Fab or scFv antibodies. Phage display vector, phagemid, is a bacterial plasmid containing sequences from a bacteriophage, in pComb3 vectors part of surface protein III gene and origin of replication. The insert coding for human antibody fragment is assembled using polymerase-chain reaction (PCR) and cloned into the vector. A wildtype phage is also needed to provide all other structural protein genes for the phage assembly. Antibody fragments are expressed as a fusion protein on the phage surface, while the antibody coding DNA is packed inside the phage particle. The antibody can be produced easily in bacteria or yeast (Barbas *et al.* 2001).

The human immunoglobulin heavy-chain variable (V_H) and light-chain variable (V_L) gene segments are amplified from B-cells by PCR and then randomly combined in another PCR, constituting diversity of the antibody library. Antibody fragments can be expressed as scFv-fragments containing only heavy and light chain variable regions, or as Fab-fragment containing also part of heavy and light chain constant region. Suitable restriction sites are created in the PCR to match the vector cloning site, and the inserts coding for antibody fragments are cloned into the phagemid vector and transformed into bacterial cells. The addition of the wildtype phage, e.g. M13 which infects transformed bacterial cells, enables the assembly of functional phage particles displaying antibody fragments on their surface. These phage particles constitute the phage display library and can be selected based on the binding properties of displayed antibody fragment. The selected phage particles are enriched and used to infect a fresh *E.coli* culture. Plasmid DNA containing the antibody coding gene segments can be isolated from bacterial cells (Barbas *et al.* 2001). The Fab cloning site in pComb3X phagemid vector and Fab display as pIII-fusion protein on phage surface is illustrated in Figure 4.

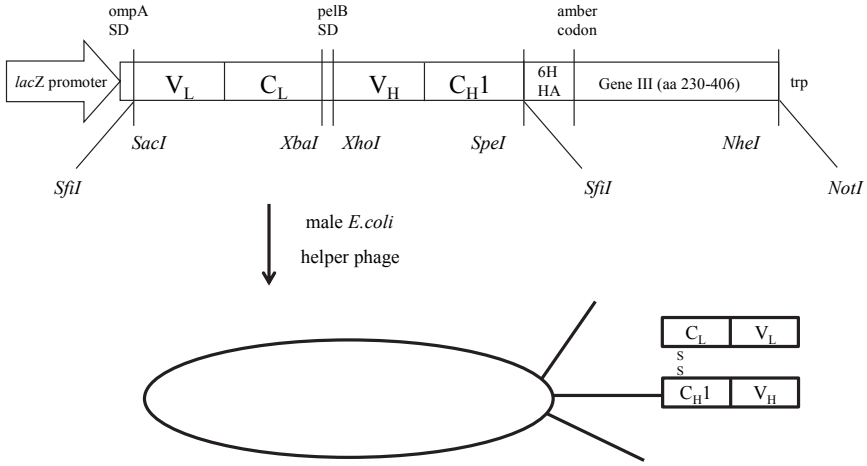

Fig. 4. Fab cloning site of pComb3X phagemid contains single *lacZ* promoter. Two Shine-Dalgarno (SD) ribosome binding sites give rise to two separate polypeptide chains. Transcription termination occurs by *trp* transcription terminator. Light chain is directed to periplasm by *ompA* leader sequence and heavy chain by *pelB* leader sequence. Hexahistide (6H) and hemagglutinin (HA) tags facilitate protein purification. Amber codon allows soluble protein production in nonsuppressor E.coli strains without phage surface protein pIII fragment. Two asymmetric *SfiI* restriction sites allows single-step directional cloning of 1500 bp Fab insert containing $V_L/C_L/V_H/C_H1$. Fab pIII-display, and also several other restriction sites have been included. When pComb3X containing Fab insert is transformed into E.coli and infected with wildtype helper phage, new phage particles containing Fab antibody as pIII-fusion protein are generated. Adapted from Barbas *et al.* (2001).

2.5.5 Fully human antibodies from transgenic mice

Genetically engineered mice expressing human antibody repertoire have been generated, and human antibody cloning has been accomplished by hybridoma technique. In the first engineered transchromosomal mice (Green *et al.* 1994, Lonberg *et al.* 1994), the mouse endogenous Ig-heavy chain and Igκ -light chain loci were disrupted, and human genes encoding Ig-heavy and Igκ-light chains were added. Since then additional V-gene segments have been included to increase the potential antibody repertoire recovered (Lonberg 2005). Several human antibodies obtained from transgenic mice are in clinical trials and some

have been approved for therapeutic use, and the amount is increasing (Nelson *et al.* 2010, Osbourn *et al.* 2003, Yamashita *et al.* 2007).

2.6 Chronic kidney disease

The patients with CKD demonstrate markers of renal damage, such as proteinuria or hematuria. The main causes for CKD are diabetes and hypertension (Alahouhala *et al.* 2012, Foley & Collins 2007). Chronic kidney disease is classified into five stages (stages 1–5) depending on the glomerular filtration rate (GFR), as shown in Table 1. CKD stage 1 is defined by kidney damage with normal or increased glomerular GFR and stage 2 by kidney damage with mildly decreased GFR. In CKD stage 3 moderately decreased GFR and in stage 4 severely decreased GFR are observed. CKD stage 5 is considered to be kidney failure with glomerular filtration rate less than 15 ml/min per 1.73m^2, or dialysis. Stages 3–5 can also be referred to as chronic renal insufficiency and treated with diet, drugs, or renal replacement therapies. End-stage renal disease patients have kidney failure (CKD stage 5) and they are treated with dialysis or kidney transplantation to preserve life (Levey & Coresh 2003, Levey *et al.* 2003, Levey *et al.* 2007).

Table 1. Classification of CKD based on glomerular filtration rate.

Stage of CKD	Definition	GFR (ml/min per 1.73m^2)
1	Kidney damage with normal or increased GFR	≥ 90
2	Kidney damage with mildly decreased GFR	60–89
3	Moderately decreased GFR	30–59
4	Severely decreased GFR	15–29
5	Kidney failure	< 15 or dialysis

Adapted from Levey *et al.* (2003).

2.6.1 Uremia and cardiovascular disease

CKD patients have a high risk for cardiovascular disease. The morbidity and mortality rates are elevated already at the initial stages of the CKD. CVD is a major cause of hospitalization and death, especially in ESRD patients (Lindner *et al.* 1974). The ESRD patients have 10–20 times higher cardiovascular mortality compared to the general population (Foley *et al.* 1998).

Traditional risk factors for cardiovascular disease include dyslipidemia, hypertension, smoking, diabetes, overweight and hyperhomocysteinemia. Other recognized risk factors that are specific for CKD are hemodynamic overload, anemia, calcium-phosphate disorders, electrolyte imbalances, chronic inflammation, oxidative stress, hypercatabolism and uremic state (Locatelli *et al.* 2003). Vascular calcification contributes to the prevalence of CVD in patients with CKD, and it is enhanced with the severity of kidney disease (Covic *et al.* 2010, Peres & Percio 2014). Several factors affect the vascular calcification, but elevated serum calcium and phosphate levels, increased calcium-phosphorous byproduct and active metabolites of vitamin D are proposed as predisposing factors for calcification in CKD patients (Razzaque 2013). Carbamylation has been suggested to promote atherogenesis in uremic patients (Apostolov *et al.* 2005, Apostolov *et al.* 2012, Basnakian *et al.* 2010, Gonen *et al.* 1985, Hörkkö *et al.* 1992, Hörkkö *et al.* 1994, Hörkkö *et al.* 1994, Hörkkö *et al.* 1995, Ok *et al.* 2005, Wang *et al.* 2007), and recent studies have provided solid evidence for increased protein carbamylation and its pro-atherogenic effect in CKD by predicting mortality in ESRD (Berg *et al.* 2013, Koeth *et al.* 2013).

2.6.2 Atherosclerosis in mouse models of uremia

Apolipoprotein E -deficient mice have hypercholesterolemia as a result of the accumulation of chylomicron remnants, very low-density lipoprotein (VLDL) and intermediate-density lipoproteins (IDL), and they spontaneously develop atherosclerosis on normal chow (Piedrahita *et al.* 1992, Plump *et al.* 1992). Atherogenesis in chronic renal failure has been studied in this mouse model after nephrectomy. Even mild renal dysfunction after unilateral nephrectomy causes increase in atherosclerotic plaque size and morphology, thus accelerating atherosclerosis (Bro *et al.* 2003, Buzello *et al.* 2003). The atherosclerosis in uremia is preceded by an upregulation of ICAM-1 in arterial endothelium and accompanied by an upregulation of ICAM-1 and VCAM-1 in the medial smooth muscle cells (Bro *et al.* 2004). Immunization with OxLDL decreases atherosclerosis in animal models (Ameli *et al.* 1996, Freigang *et al.* 1998, Nilsson *et al.* 1997, Palinski *et al.* 1995, Zhou *et al.* 2001). However, OxLDL immunization was not able to prevent atherogenesis in uremic mice, suggesting that pro-atherogenic and pro-inflammatory influence of uremia overrides the anti-atherogenic effect of OxLDL immunization (Pedersen *et al.* 2010). Chronic renal failure can be induced in mice with electrocoagulation of the kidney cortex

(Gagnon & Duguid 1983) or with surgical removal of the kidney. Study of Apostolov *et al.* (2010) investigated the effect of direct urea administration in drinking water on atherogenesis in apoE$^{-/-}$ mice after unilateral nephrectomy. The addition of 2% urea in drinking water was able to elevate plasma urea levels to the same level as CRF mouse model (80% right kidney electrocoagulation and left kidney removal) without any other factors or toxins related to chronic renal failure. Elevated plasma urea induces LDL carbamylation in urea-fed mice which associates with enhanced atherogenesis similarly to the CFR model (Apostolov *et al.* 2010). Carbamylated proteins have shown to also accumulate in tissues demonstrated in nephrectomised C57BL/6J mice without atherosclerosis (Pietrement *et al.* 2013).

3 Aims of the study

The present study was designed to investigate humoral immune response to carbamyl-epitopes and antibody cross-reaction between carbamylated and oxidation-specific epitopes. Patients with chronic kidney disease have higher plasma urea levels, and therefore more carbamylation of plasma proteins. The uremic patients also have a higher prevalence of cardiovascular disease which cannot be only explained by the traditional risk factors, for example elevated LDL cholesterol level.

The hypothesis of the current thesis study was that LDL carbamylation is more extensive in uremic patients, and induces formation of antibodies to carbamyl-epitopes. Humoral immune response to this modified form of LDL could have a crucial role in the development of atherosclerosis. Carbamylated LDL share many similar properties with oxidized LDL, and humoral immune response cross-reactivity is also possible. The specific study questions were:

1. Are antibodies to carbamylated proteins present in human plasma, and is it possible to clone human monoclonal anti-carbamyl antibodies?
2. What are the binding properties and functionality of human anti-carbamyl-antibodies?
3. Is carbamylated LDL immunogenic in mice, and does immunization with carbamyl-LDL affect atherogenesis in $LDLR^{-/-}$ mouse strain?

4 Materials and Methods

4.1 Cells, animals and human study subjects

4.1.1 Cells and animals

The cells and animals used in the studies included in this thesis are listed in Table 2.

Table 2. Cells and animals used in this thesis.

Cell and animals	Original publication	Description
Bacteria		
XL1-Blue	II	*E.coli* strain for phage display library transformation
TOP10F'	II	Non-suppressor *E.coli* strain for production of soluble Fab
BL21(DE3)	II	*E. coli* strain for efficient protein production
Cells		
Jurkat, clone E6-1	II, III	Human acute T-cell leukemia cell line (ATCC® TIB-152™)
J774A.1	II	Mouse reticulum cell sarcoma macrophage cell line (ATCC® TIB-67™)
Mice		
$LDLR^{-/-}$	I,II	LDL receptor-deficient mouse model, develops high fat diet-induced atherosclerosis (C57BL/6J background B6.129S7-*Ldlrt*^{m1Her})
C57BL/6	I, II	Genetic background control for $LDLR^{-/-}$ mice

Bacterial cultures

E.coli XL1-Blue was cultured in LB media (containing 10 g/l tryptone, 5 g/l yeast extract and 10 g/l NaCl, pH 7.0). SOC media (containing 20 g/l tryptone, 5 g/l yeast extract, 0.5 g/l NaCl, 186 mg/l KCl, 10 mmol/l $MgCl_2$ and 20 mmol/l glucose, pH 7.0) was used after transformation to obtain maximal transformation efficiency. After transformation bacterial cultures were grown in Super Broth (SB) during phage display library selection. SB contains 10 g/l 3(N-morpholino)propanesulfonic acid (MOPS), 30 g/l tryptone and 20 g/l yeast extract, pH 7.0. Soluble Fab antibodies were produced in *E.coli* TOP10F' and in *E.coli* BL21(DE3) for large scale protein production. Both of these bacterial strains were cultured in SB medium.

Cell cultures

Human Jurkat T-cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 10 mmol/l HEPES, 1 mmol/l sodium pyruvate, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Mouse J774A.1 macrophages were grow in Dulbecco's modified Eagle medium (DMEM) with 4,5 g/l glucose, 110 mg/l sodium pyruvate and 110 mg/l L-glutamine supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were grown in a humified atmosphere with 5% CO_2 at +37 °C.

Mouse immunization and high fat diet protocol

Thirty-one $LDLR^{-/-}$ mice were divided into three immunization groups; phosphate buffered saline (PBS) n = 11, native mouse LDL n = 10, and carbamylated mouse LDL n = 10. The primary immunizations were given subcutaneously (s.c.) with 50 µg LDL. Four booster immunizations were given intraperitoneally (i.p.) with 25 µg of LDL at weeks 3, 6, 12 and 16 of the study. The mice were fed regular chow diet (4.4% fat and 0.01% cholesterol) during the first 16 weeks. High fat diet (HFD; 21.2% fat and 0.2% cholesterol, 42% of calories from fat) to induce atherosclerosis was initiated at week 17. The immunizations were continued (25 µg, i.p.) once a month during the high fat diet, at weeks 20, 24 and 29 of the study. No adjuvants were included in this study. Blood samples were collected at the beginning of the study (week 0), after immunizations and before HFD (week 16), and after immunizations and HFD (week 35) from hind leg vein. The mice were sacrificed by CO_2 inhalation during the weeks 36 and 37 of the study, and final blood samples were collected immediately after sacrifice from posterior *vena cava*. The scheme is presented in Figure 5.

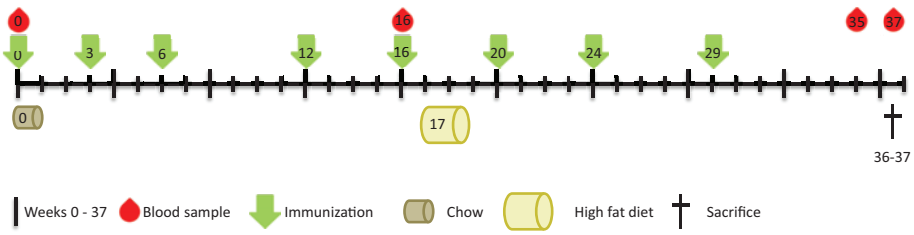

Fig. 5. Mouse study timeline.

4.1.2 Human study subjects

The collection of human blood samples for these studies was approved by the Ethics Committee of Oulu University Hospital and the studies followed the Declaration of Helsinki. The subjects participated voluntarily and gave informed written consent for the studies.

Hemodialysis patients and controls

Thirty-nine patients with chronic kidney disease receiving hemodialysis treatment were recruited from the Dialysis Unit of Oulu University Hospital. Blood samples were collected into ethylenediaminetetraacetic acid (EDTA) containing tubes before and after the dialysis session during the second dialysis treatment of the week. Forty-two healthy controls with normal kidney function were enrolled from Clinical Research Center of Oulu University Hospital. One blood sample was collected from each participant into EDTA tube. The controls were not matched for age and sex. The clinical characteristics of the study subjects are represented in Table 3.

Table 3. Clinical characteristics (mean ± SD) of hemodialysis patients and healthy controls. Modified from the original publication I Supplemental Table S1 with permission from Mary Ann Liebert.

Variable	Hemodialysis patients (n = 39)	Controls (n = 42)
Gender		
Female	11	26
Male	28	16
Age (years)	59.2 ± 17.8	36 ± 8.8
Total cholesterol (mmol / l)	3.6 ±1.1	4.9 ± 0.9
HDL cholesterol (mmol / l)	1.2 ± 0.5	1.5 ± 0.3
LDL cholesterol (mmol / l)	1.8 ± 0.7	2.9 ± 0.8
Triglycerides (mmol / l)	1.6 ± 0.8	1.0 ± 0.4
Glucose (mmol / l)	6.9 ± 2.7	5.3 ±0.6
Creatinine (µmol / l)	678.5 ± 207.0	68.9 ± 10.4
Albumin (g / l)	38.6 ± 3.6	45.5 ± 2.5
Urea (mmol / l)	19.0 ± 4.5	5.2 ± 1.2
Urea, after dialysis (mmol / l)	6.2 ± 1.5	
Smoking status		
Non-smoker	19	34
Ex-smoker	9	1
Smoker	11	7

Human plasma for low-density lipoprotein isolation

Blood samples were collected from healthy volunteers, and EDTA-plasma was used for isolation of low-density lipoprotein fraction by gradient ultracentrifugation.

Peripheral blood mononuclear cells for generation of phage display library

Blood samples were collected from four healthy volunteers for phage display library construction. Blood was drawn into EDTA-plasma tubes and peripheral blood mononuclear cells were isolated using Ficoll-Paque density gradient centrifugation and used for isolation of total ribonucleic acid (RNA).

Peripheral blood mononuclear cells for EBV-transformation

Blood sample from one volunteer was collected into BD Vacutainer® CTP™ -tubes and mononuclear cells were separated by centrifugation and used for EBV transformation.

4.2 Laboratory methods

The main laboratory methods used in the studies described in this thesis are summarized in Table 4 and described below. Further information on the methods can be found in the original publications I, II and III.

Table 4. Methods used in the studies.

Method	Original publication
Low-density lipoprotein isolation	I, II, III
Low-density lipoprotein / albumin modification	I, II, III
Chemiluminescence immunoassay	I, II, III
Competitive immunoassay	I, II, III
Determination of mouse atherosclerosis	I
Immunostaining	I, II
Phage display library construction	II
Fab antibody production	II
Flow cytometry assay of apoptotic cells	II, III
Macrophage uptake assay	II

4.2.1 Low-density lipoprotein isolation

Human low-density lipoprotein fraction (1.019–1.063 g/ml) was isolated from pooled plasma of healthy donors by sequential density gradient ultracentrifugation (Havel *et al.* 1955). Mouse LDL was isolated similarly from pooled plasma of $LDLR^{-/-}$ mice fed with high fat diet.

4.2.2 Low-density lipoprotein and albumin modification

Butylated hydoxytoluene (BHT, 20 μmol/l) and ethylenediaminetetraacetic acid (EDTA, 0.27 mmol/l) were added to the freshly isolated LDL fraction to minimize oxidation prior to the modifications. Antioxidants were not added to the LDL that was used for copper-oxidation. After modifications, LDL and albumin were

dialyzed against PBS with 0.27 mmol/l EDTA, and sterile filtered (0.2 μm). The extent of modifications were verified with 2,4,6-trinitrobenzenesulfonic acid (TNBS) method (Habeeb 1966).

Carbamylation

In vitro carbamylation of LDL was performed with potassium cyanate (KCNO) (Weisgraber *et al.* 1978). LDL (approximately 2 mg/ml) was diluted 2:3 with 0.3 mol/l $Na_2B_4O_7$ -buffer, pH 7.0 and KCNO was added (20 mg per mg of LDL). The carbamylation was carried out at +37 °C for 6 hours. Albumin was carbamylated similarly, but at higher concentration (5–10 mg/ml) and for 24 hours.

Malondialdehyde (MDA)- and malondialdehyde acetaldehyde (MAA)-modification

For MDA-modification, fresh MDA solution was prepared (0.5 mol/l 1,1,3,3-tetramethoxypropane malonaldehyde-bis(dimethyl acetal) in 0.6% HCl) and incubated at +37 °C for 10 minutes. The pH was adjusted to 6.0–7.0 with NaOH. 0.5 mol/l MDA (150μl) was used to modify LDL (1 mg) for 3 hours at +37 °C (Palinski *et al.* 1990).

For MAA-modification, fresh MDA (0.5 mol/l, pH 4.8) was prepared. 310 μl PBS, 140 μl 20% acetaldehyde, 5 mg LDL and 300 μl 0.5 mol/l MDA pH 4.8 were mixed in order. The pH was readjusted and the mixture was incubated for 2 hours at +37 °C (Hill *et al.* 1998, Tuma *et al.* 1996). MDA- and MAA-modifications were done similarly for albumin (bovine serum albumin fraction V, BSA or mouse serum albumin, MSA).

Copper-oxidation

LDL without BHT was extensively dialyzed to remove EDTA. LDL (1 mg/ml) was oxidized with 4 mM $CuSO_4$ at +37 °C for 24 hours (Palinski *et al.* 1990). The reaction was stopped by addition of EDTA to a 200 μmol/l final concentration.

4.2.3 Chemiluminescence immunoassay

Chemiluminescence based immunoassay was used to detect antibodies binding to specific antigen, or for detection of antibody levels in plasma samples (Nissinen *et al.* 2011, Turunen *et al.* 2012, Veneskoski *et al.* 2011, Wang *et al.* 2013). The assays were performed on 96-well plates coated with antigen, or antibody, diluted in PBS with 0.27 mmol/l EDTA overnight at +4 °C. The wells were blocked with 0.5% gelatin in PBS with 0.27 mmol/l EDTA for 30 minutes. The primary antibody, or plasma sample, diluted in 0.5% gelatin in PBS with 0.27 mmol/l EDTA was added and incubated in wells for 1 hour at room temperature or overnight at +4 °C. The amount of bound molecules was detected with alkaline phosphatase conjugated secondary antibody or alkaline phosphatase conjugated Neutravidin for biotinylated primary antibodies diluted in 0.5% gelatin in tris buffered saline (TBS) with 0.27 mmol/l EDTA and 1 mmol/l $MgCl_2$. The plates were washed after each step with PBS with 0.27 mmol/l EDTA using automated plate washer. Finally, LumiPhos530 substrate for alkaline phosphatase was added and incubated for 90 minutes. The chemiluminescence was measured with Wallac Victor^3 as relative light unit in 100 ms (RLU / 100 ms).

4.2.4 Competitive immunoassay

The specificity of antibodies was tested using liquid-phase competitive immunoassay. Antibody or plasma dilutions were incubated overnight at +4 .T°C in the presence or absence of competitors (0–200 µg/ml) followed by centrifugation for 30 minutes at 16000 × g and +4 °C to pellet the immunocomplexes. Antibodies remaining in the solution were analyzed using chemiluminescence immunoassay or flow cytometry assay.

4.2.5 Determination of mouse atherosclerosis

Mouse atherosclerosis was determined from LDLR$^{-/-}$ mice fed with high fat diet using two different methods, *en face* analysis of the whole aorta and determination of plaque area from aortic-origin heart cross-sections (Kellokoski *et al.* 2009, Tangirala *et al.* 1995, Turunen *et al.* 2012).
The mice were sacrificed by CO_2 inhalation and the blood was collected with EDTA-primed needle and syringe from posterior *vena cava*. The mice were first perfused with PBS with 1 µg/ml BHT and then fixed with formalin-sucrose (10%

formalin, 5% sucrose, 3 µmol/l EDTA and 20 µmol/l BHT, pH 7.4) through the left ventricle, each for 10 minutes. The aortas were cleaned from surrounding tissues under preparation microscope and detached. Hearts were also removed, and kept in 10% formalin. The aortas were stained with Sudan IV, cut open and pinned onto paraffin plates. The stained aortas were imaged with QImaging MicroPublisher 5.0 RTV camera, and atherosclerotic plaque visualized by red staining was quantified with MCID Core 7.0 analysis software. The aortic area was determined manually, and red staining automatically. Atherosclerotic plaque area was expressed as total plaque area in μm^2, or as percentage of plaque area from total aortic area.

The formalin-fixed mouse hearts were embedded in paraffin and 5 µm cross-sections were cut from the apex towards the aortic valves. The cross-sections were collected once the first aortic valve appeared, until the last aortic valve was visible. Three cross-sections were collected onto one slide and every third slide was stained with hematoxylin-eosin. The stained cross-sections were analyzed using QImaging MicroPublisher 5.0 RTV camera attached to the Leiz Aristoplan microscope and MCID Core 7.0 software. In each cross-section the aortic area, and also the plaque areas under each valve, were determined manually. The peak area value for atherosclerotic plaque was determined individually for each valve. Atherosclerotic area was expressed as a percentage of the total area.

4.2.6 Immunostaining of mouse heart aortic-origin cross-sections

Aortic-origin mouse heart cross-sections (5 µm) were used for immunostaining with polyclonal rabbit IgG (I) or monoclonal human Fab (II) antibodies binding to carbamyl-epitopes. Goat-on-Rodent HRP-polymer kit was used in the staining. Sodium citrate (10 mmol/l, pH 6.0) was used for antigen retrieval in 10-minute treatment near boiling point in microwave oven. Endogenous peroxidase activity was quenched with peroxidase block.

4.2.7 Phage display antibody cloning

The phage display library was generated using previously published human Fab-primers (Andris-Widhopf *et al.* 2001, Barbas *et al.* 2001) and phagemid vector pComb3X obtained from Dr. C.F. Barbas, Scripps Research Institute, La Jolla, San Diego, California. The primer sequences are shown in Table 8 and Table 9 in the Appendix. Constant regions were amplified using templates cloned in

pComb3XTT and pComb3Xλ vectors. Human peripheral blood lymphocytes were used as a starting material for amplification of variable regions of antibody heavy- and light-chain fragments. Total RNA was isolated from peripheral blood lymphocytes and complementary DNA (cDNA) was synthetized using oligo(dT)18 primers and the first strand cDNA synthesis kit. The cDNA was used as a template in first round of PCR.

The first round of PCR consists of four different PCR reactions. 350–400 bp regions in heavy chain constant, heavy chain variable, light chain constant and light chain variable regions were amplified with a specific set of primers. Altogether 12 primer pairs were used for amplification of heavy chain, four primer pairs for κ-light chain and nine primer pairs for λ-light chain variable region. One primer pair was used for amplification of each constant region from plasmids. The second round of PCR created 750–800 bp overlap extension PCR products. The first round variable- and constant-region PCR products were combined in equal ratios and served as a template in second round PCR. Three different reactions were performed: one for heavy chain and two for light chain (κ and λ separately). The final third round PCR resulted in a 1500 pb full-length Fab coding fragment. The full-length Fab-coding PCR product and phagemid vector pComb3X were digested with restriction endonuclease *SfiI* which allows directional cloning of the insert into pComb3X phagemid vector. The *SfiI* digested antibody-coding PCR-products were ligated into pComb3x vector digested with the same restriction endonuclease. The cloning site of pComb3X phagemid with Fab insert is shown in Figure 4.

The ligation mixture was transformed into *E.coli* XL1-Blue by electroporation using GenePulser electroporator and cuvette with 0.2 cm gap to achieve the highest transformation efficiency. The transformed *E.coli* were cultured overnight and rescued with wildtype bacteriophage VCSM13 that provides all structural proteins for phage particle assembly. Phage particles were precipitated from overnight culture with 4% (w/v) PEG-8000 and 3% (w/v) NaCl, and pelleted with centrifugation at 15000 × g for 15 minutes. Selection of phage particles binding to the antigen of interest, carbamyl-LDL, was performed in five rounds of panning. Human carbamyl-LDL was immobilized onto 96-well plate and precipitated phage pool was added to the wells for 4 hours at 37 °C. Non-binding phage particles were washed away, and binding phages were detached from the wells with acidic buffer. A fresh *E.coli* culture was infected with eluted phage particles, and grown overnight until precipitated again. This was repeated for five rounds, and enrichment of carbamyl-LDL binding phage particles was

monitored with chemiluminescent immunoassay using anti-M13 antibody binding to phage particles. The carbamyl-LDL binding phage particles were enriched until the fourth round. *E.coli* infected with precipitated phage pool after five rounds of panning was plated on LB-agar. Single colonies were picked and grown overnight. Plasmid DNA was isolated for sequencing and supernatant was collected for testing the Fab-pIII fusion protein binding to carbamyl-LDL in chemiluminescent immunoassay.

4.2.8 Fab antibody production and purification

Soluble Fab106 antibody was produced in *E.coli* strains TOP10F' and BL21(DE3). These non-supressor strains allow the production of soluble Fab antibody without phage surface protein. *E.coli* harboring the antibody-coding plasmid was grown on an LB-plate with 100 mg/ml ampicillin. One colony was transferred into 10 ml of SB-medium and 100 mg/ml of ampicillin and cultured overnight. The overnight culture was then diluted into 1 l of SB medium with 100 mg/ml ampicillin and bacteria were grown until OD_{600} reached the absorbance of 1.0. Isopropyl-thiogalactosidase (IPTG) was added to 1–2 mmol/l final concentration to induce *lac* promoter and protein production, and the culture was incubated at 37 °C for 20–24 hours with vigorous shaking of 250–300 rpm. The cells were pelleted by centrifugation at 3000 × g for 30 minutes at 4 °C, and the supernatant was collected for protein purification. The supernatant was filtered with 0.22 µm filter and concentrated to 1/10 of original volume using concentrator with 10 kDa MWCO. The Fab antibody was purified from the concentrated supernatant with commercial 1 ml protein G-column attached to a peristaltic pump according to the manufacturer's instructions. Approximately 2 mg of Fab antibody was obtained from 1 l bacterial culture.

4.2.9 Macrophage uptake assay

Macrophage uptake assay was performed with labelled modified LDL in J774A.1 mouse macrophages (Veneskoski *et al.* 2011). 3H-labelled carbamyl-LDL or IRDye 800-conjugated MDA-LDL was used in the assay.

4.2.10 Flow cytometry assay of apoptotic cells

Apoptosis was induced in Jurkat cells in Petri dish with ultraviolet (UV)-irradiation (51 mJ/m^2) with Stratalinker UV-crosslinker. Flow cytometry assays detected either the direct binding of antibodies to apoptotic Jurkat cells or the competition of antibodies with soluble antigens (Tuominen *et al.* 2006, Turunen *et al.* 2012, Veneskoski *et al.* 2011). Flow cytometry assays were performed using FACS Calibur flow cytometer.

4.2.11 Statistical analysis

Statistical analyses were performed with the SPSS Statistics (19.0) software. The results for continuous variables are presented as mean ± standard deviation (SD). The differences between the groups were analyzed using parametric Student's t-test or nonparametric Mann-Whitney U -test depending on the distribution of the study population. Associations were analyzed using Spearman's correlation coefficient. P-value < 0.05 was regarded as statistically significant. P-values are marked in the figures with asterisks as follows: $*$ $P < 0.05$, $**$ $P < 0.01$, $***$ $P < 0.001$.

5 Results

5.1 Human plasma contains antibodies to carbamylated proteins (I)

High plasma urea levels are known to cause carbamylation in patients with CKD. IgG and IgM antibody levels to carbamyl-LDL and carbamyl-albumin were detected in plasma samples of hemodialysis patients, and even in healthy controls. Hemodialysis patients had more IgG antibodies binding to carbamylated proteins, both LDL and albumin, compared to the controls (Figure 6). There was no statistically significant difference in IgM antibody levels between the study groups (data shown in the original publication I, Figure 9C-D).

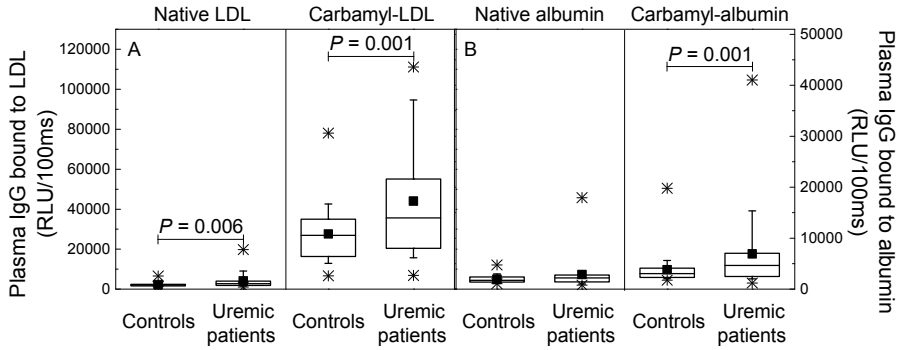

Fig. 6. Human plasma IgG antibodies to carbamyl-epitopes in uremic patients (n = 39) and control subjects with normal plasma urea levels (n = 42). (A) Plasma IgG levels (1:500) to native LDL and carbamyl-LDL. (B) Plasma IgG levels to native albumin and carbamyl-albumin. The box plots represent 25%, 50% and 75%, and the whiskers represent 10% and 90% distribution of values within the groups. The asterisks are minimum and maximum values and the squares are the mean values. RLU = relative light units. Modified from the original publication I Figure 9 and reprinted with permission from Mary Ann Liebert.

In addition to urea, smoke exposure is also known to promote carbamylation *in vivo*. IgG antibody levels to carbamyl-LDL were further investigated separately in current smokers and non-smokers within study groups. The IgG level to carbamyl-LDL was highest in smoking dialysis patients and lowest in non-smoking controls. The difference in IgG levels between smokers and non-smokers were statistically significant in both study groups (Figure 7).

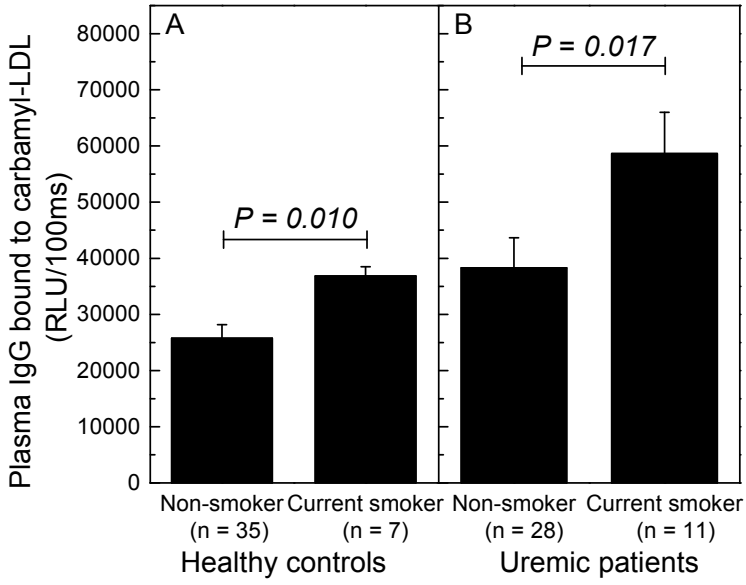

Fig. 7. Plasma IgG levels to carbamyl-LDL in smoking and non-smoking control subjects (A), and in smoking and non-smoking uremic patients (B). Triplicate measurements for each human sample were made and mean (SD) within a group is shown. RLU = relative light units. Reprinted from the original publication I Figure 10 with permission from Mary Ann Liebert.

All the study subjects were divided into three groups according to the smoking status (smoker, ex-smoker or non-smoker) and into quartiles according to the plasma urea concentration. There was a clear trend in IgG antibody level to carbamyl-LDL depending on the smoking status and urea level. Plasma IgG to carbamyl-LDL was higher in smokers compared to non-smokers or ex-smokers, and also higher in ex-smokers compared to non-smokers. IgG level to carbamyl-LDL also rose with increasing urea level, shown as urea quartiles (Figure 8).

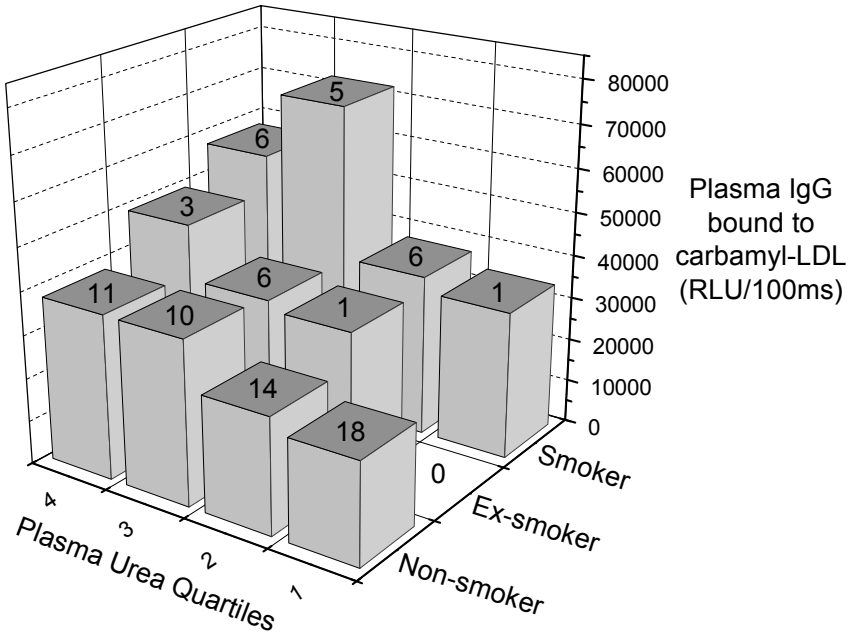

Fig. 8. Urea quartile vs. IgG antibody level to carbamyl-LDL vs. smoking in all study subjects (n = 81). The number of study subjects in each category is shown on top of the column. The lowest IgG antibody levels to carbamyl-LDL were observed in non-smokers with lowest urea quartile, and elevated with increasing plasma urea and smoking. RLU = relative light units.

5.2 Association of antibody response to carbamyl- and oxidation-specific epitopes in humans (II, III)

Human plasma antibody levels to oxidation-specific epitopes, CuOxLDL, MDA-LDL and MAA-LDL, and to MDA-BSA and MAA-BSA, were also measured from human plasma samples and association with antibodies to carbamyl-LDL was investigated. Human IgG and IgM antibodies bound to carbamyl-LDL associated with antibodies bound to oxidation-specific epitopes on LDL. Further, antibodies bound to carbamyl-albumin associated with antibodies bound to oxidation-specific epitopes in albumin. Strong, positive association was seen in healthy subjects (II) as well as in hemodialysis patients (Table 5).

Table 5. Antibodies to carbamyl-epitopes associate with antibodies to oxidation-specific epitopes. Modified from the original publication II Figure 1 with permission from John Wiley & Sons.

Association in antibody binding	Healthy controls		Hemodialysis patients	
	ρ	*P*	ρ	*P*
IgG				
Carbamyl-LDL vs. CuOxLDL	0.759	< 0.001	0.889	< 0.001
Carbamyl-LDL vs. MDA-LDL	0.857	< 0.001	0.934	< 0.001
Carbamyl-LDL vs. MAA-LDL	0.696	< 0.001	0.880	< 0.001
Carbamyl-BSA vs. MDA-BSA	0.829	< 0.001	0.964	< 0.001
Carbamyl-BSA vs. MAA-BSA	0.647	< 0.001	0.698	< 0.001
IgM				
Carbamyl-LDL vs. CuOxLDL	0.833	< 0.001	0.806	< 0.001
Carbamyl-LDL vs. MDA-LDL	0.738	< 0.001	0.792	< 0.001
Carbamyl-LDL vs. MAA-LDL	0.769	< 0.001	0.776	< 0.001
Carbamyl-BSA vs. MDA-BSA	0.820	< 0.001	0.594	< 0.001
Carbamyl-BSA vs. MAA-BSA	0.735	< 0.001	0.450	0.004

The binding specificity of human plasma antibodies bound to carbamyl-LDL was investigated in competitive immunoassays (data shown in the original publication II, Figure 2). Soluble carbamyl-LDL and MDA-LDL competed for binding of plasma IgG and IgM antibodies to carbamyl-LDL and to MDA-LDL. Carbamyl-BSA, MDA-BSA and MAA-BSA competed for plasma IgG binding to carbamyl-BSA and MDA-BSA. Plasma IgM levels to carbamyl-BSA and MDA-BSA were low, and competition was not analyzed. MDA- and MAA-adducts competed more efficiently for human plasma IgG and IgM binding to immobilized carbamylated antigens than carbamylated proteins.

IgG and IgM antibody levels were normalized with the total IgG and IgM concentration (data shown in the original publication III, Figure 3). After normalization, hemodialysis patients had more IgG antibodies bound to carbamyl-LDL as observed earlier (I), but also higher IgG antibody binding to CuOxLDL and MAA-LDL. There were no differences in relative IgM levels to carbamyl-LDL or oxidation-specific epitopes between hemodialysis patients and controls (data shown in the original publication III, Figure 3).

5.3 Cloning of human antibodies to carbamyl-adducts (II)

Human monoclonal Fab antibodies against carbamyl-epitopes were cloned from phage display library generated from healthy humans. Several individual clones were selected, based on the binding to immobilized human carbamyl-LDL. Sequence analyses of ten clones are represented in Table 6. The clones originated mostly from the V_H3 antibody gene family and demonstrated surprisingly high variable-region homology to germline. One clone, named IFP-02_106, from κ-library was chosen for further studies based on high binding to carbamyl-LDL, high production rate of soluble Fab in *E. coli*, and high V-region germline homology.

Table 6. Sequence analysis of ten human Fab clones selected for carbamyl-LDL binding. Modified from the original publication II Table 1 with permission from John Wiley & Sons.

Fab clone	V(D)J usage	CDR3 amino acid sequence	V-region identity to germline
κ1			
heavy	V_H3-33*01 / D_H6-25*01 / J_H4*02	ARDGRVRRGGAGAQRGLDY	100.0%
light	Vκ3-20*01 / Jκ1*01	QQYGGSPW	97.3%
κ2 [1]			
heavy	V_H3-33*01 / D_H6-25*01 / J_H4*02	ARDGRVRRGGAGAQRGLDY	100%
light	Vκ3-20*01 / Jκ1*01	QQYGGSPW	91.8%
κ5			
heavy	V_H3-33*01 / D_H3-10*01 / J_H4*02	ARGSGSNFWRPYYFDN	86.1%
light	Vκ1-39*01 / Jκ1*01	QQTYNTRG	90.7%
κ10			
heavy	V_H3-23*01 / D_H3-3*01 / J_H4*02	AKVEGPLDTIFGVVIIPEFDY	100.0%
light	Vκ1-39*01 / Jκ3*01	QQSYHIPFN	89.9%
κ11			
heavy	V_H4-39*01 / D_H1-26*01 / J_H4*03	ARLVGSTTGYFDP	91.1%
light	Vκ1-5*03 / Jκ1*01	LQDYSYPRT	90.6%
λ1			
heavy	V_H3-33*01 / D_H3-3*01 / J_H4*02	AKDLAVVQMGTFDY	99.2%
light	Vλ3-1*01 / Jλ3*02	QAWDSNIAV	57.2%
λ4			
heavy	V_H3-33*01 / D_H2-02*01 / J_H4*02	ARDYIVVVPAAYGGIDY	93.2%
light	Vλ2-14*03 / Jλ2*01	SSYSRSSTLVL	73.6%
λ5			
heavy	V_H3-23*01 / D_H5-18*01 / J_H4*02	ARRKHLRFRGHRYFDY	97.0%
light	Vλ3-1*01 / Jλ2*01	QAWDNNIVV	93.2%
λ6			
heavy	V_H3-33*01 / D_H2-8*01 / J_H4*02	ARARGRRGMVLLLWT	93.5%
light	Vλ10-54*01 / Jλ3*02	SAWDNNLSAWV	90.7%
λ11			
heavy	V_H3-66*02 / D_H3-3*01 / J_H4*03	ARDFWSGYFDY	93.5%
light	Vλ2-28*01 / Jλ2*01	MQALQTYTF	100.0%

[1] Clone IFP-02_106 (original publication II)

5.4 Cross-reactivity of human anti-carbamyl-Fab with malondialdehyde-adducts (II)

The Fab clone IFP-02_106 was produced in *E.coli*, purified with protein G affinity column, and named Fab106. The purified Fab was characterized in detail using direct immunoassay and competitive immunoassay (Figure 9), and also Western blot and Biacore interaction assay (data shown in the original publication II, Figures 3 and 4).

In direct binding assay (Figure 9A) Fab106 showed increase in binding dose-dependently to carbamyl-LDL but also to oxidation-specific epitopes MDA-LDL, MAA-LDL and CuOxLDL. Similar binding was detected for carbamyl-, MDA- and MAA-BSA, but not for PC-BSA. This implies that binding to CuOxLDL resulted from MDA-epitopes generated during copper-oxidation of LDL. No binding to native LDL or BSA was detected.

Competitive liquid phase immunoassay (Figure 9B) was performed to test the binding specificity of Fab106 to carbamyl-modification. Fab106 binding to carbamyl-LDL was competed out by the addition of soluble carbamyl-LDL or carbamyl-BSA, but not by native LDL or BSA, suggesting specific binding to carbamyl-epitope and not to carrier protein. Also MDA- and MAA-modified LDL and BSA competed out Fab106 binding to carbamyl-LDL, demonstrating cross-reaction between carbamyl- and MDA-/MAA-epitopes. CuOxLDL also competed for Fab106 binding, whereas PC-BSA did not, verifying the involvement of MDA-epitopes, not PC-epitopes, in CuOxLDL.

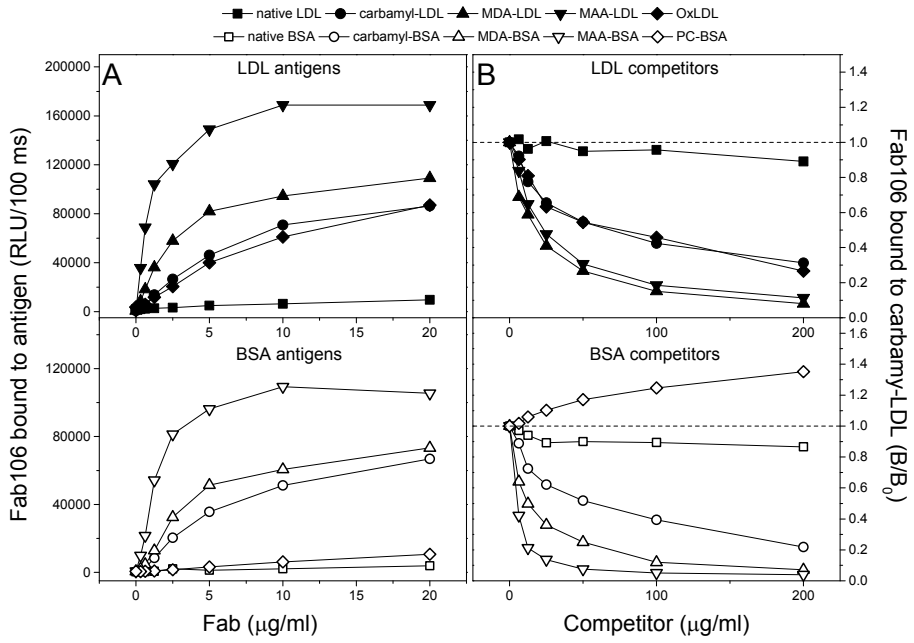

Fig. 9. Fab106 binding to carbamyl- and oxidation-specific epitopes in direct binding assay (A) and competitive liquid phase immunoassay to demonstrate Fab106 binding specificity (B). Increasing amount of Fab106 (0–20 µg/ml) bound to native LDL, carbamyl-LDL, MDA-LDL, MAA-LDL and OxLDL (A, upper panel) or to native BSA, carbamyl-BSA, MDA-BSA, MAA-BSA and PC-BSA (A, lower panel). Fab106 binding to immobilized carbamyl-LDL was tested when native BSA, carbamyl-LDL, MDA-LDL, MAA-LDL and OxLDL and also native BSA, carbamyl-BSA, MDA-BSA, MAA-BSA and PC-BSA were used as competitors (B). RLU = relative light unit. Modified from the original publication II Figure 3 and reprinted with permission from John Wiley & Sons.

The Fab106 binding to the epitopes demonstrated was confirmed with Western blot. Fab106 bound to carbamyl-BSA, MDA-BSA and MAA-BSA in Western blot, but no binding to native BSA was observed (data shown in the original publication II, Figure 3C-D).

The steady state binding affinity of Fab106 to the antigens was tested by surface plasmon resonance analysis (Table 6). The strongest binding affinity of Fab106 was observed to MDA-LDL and MAA-LDL. Binding to carbamyl-LDL was also high, when compared to native LDL. Fab106 bound to modified BSA, but with slightly weaker affinity when compared to modified LDL. Binding affinities to native LDL and native BSA were very weak, as shown in Table 7.

Table 7. Steady-state affinity K_D (mol/l) values for immobilized Fab106 binding to soluble antigens in Biacore interaction analysis. Modified from the original publication II Figure 4 with permission from John Wiley & Sons.

Antigen	Affinity K_D (mol/l)
BSA	
Native BSA	9.70e-2
Carbamyl-BSA	1.58e-4
MDA-BSA	5.09e-4
MAA-BSA	3.28e-5
LDL	
Native LDL	8.08e-1
Carbamyl-LDL	2.13e-5
MDA-LDL	5.24e-6
MAA-LDL	4.07e-6

5.5 The functionality of human monoclonal Fab (II)

The ability of monoclonal Fab106 antibody to inhibit the uptake of modified LDL by J774A.1 mouse macrophages *in vitro* was investigated. Fab106 was able to significantly inhibit the uptake of carbamyl-LDL (by 63%) and MDA-LDL (by 33%) as shown in Figure 10.

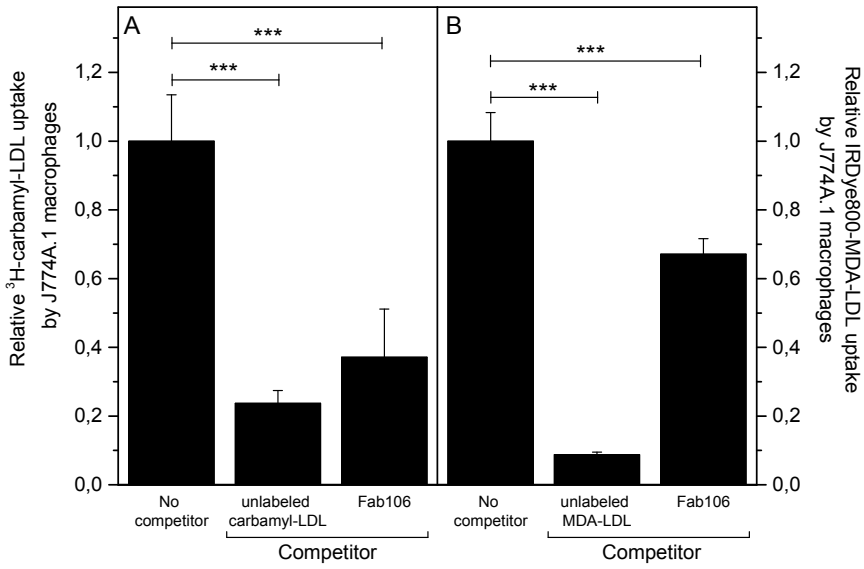

Fig. 10. The uptake assay of carbamyl-LDL and MDA-LDL by J774A.1 by mouse macrophages. Uptake of ^{3}H-labelled carbamyl-LDL (A) and IRDye800-labelled MDA-LDL (B) was tested in the presence and absence of Fab106. 30x or 50x excess of unlabelled carbamyl- or MDA-LDL were used as an assay control. Reprinted from the original publication II Figure 7 and with permission from John Wiley & Sons.

The human Fab106 also bound to epitopes found in advanced mouse atherosclerotic lesions (Figure 11).

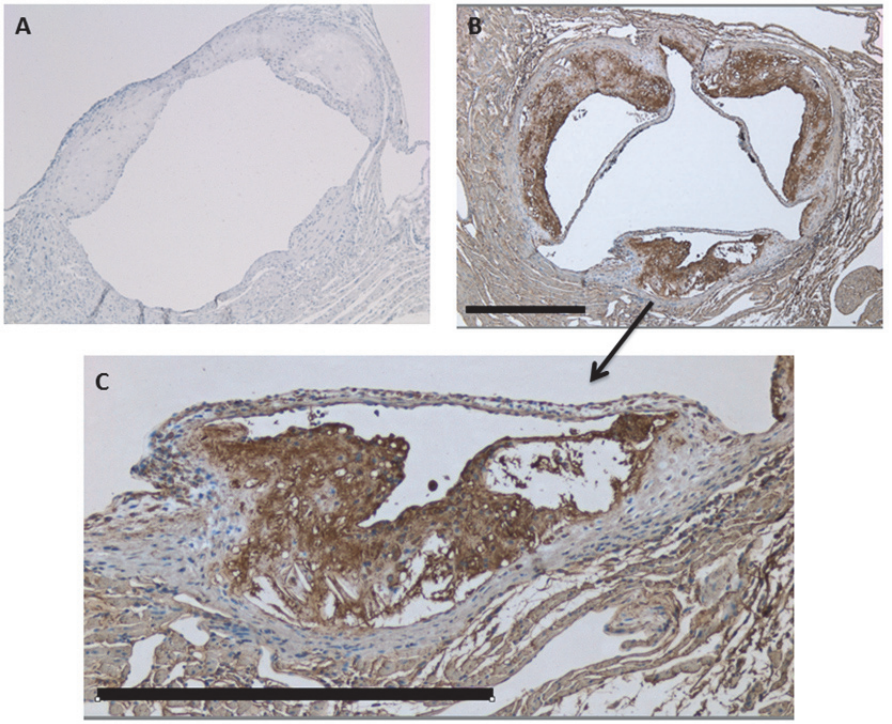

Fig. 11. Fab 106 antibody binds to atherosclerotic lesions in mouse heart aortic-origin cross-section in immunohistochemical staining. Secondary antibody control (A) and Fab106 staining (B,C). Magnification 5× (A, B) and 10× (C), scale bar = 400 µm. Reprinted from the original publication II Figure 8 with permission from John Wiley & Sons.

5.6 Human antibodies secreted by EBV-immortalized B-cells (III)

Human B-cells immortalized with Epstein-Barr virus were screened for binding to carbamyl-LDL. Both IgG and IgM antibody secreting clones were found, and two IgG and two IgM secreting clones were selected. All these clones bound carbamyl-LDL, but also MDA-LDL and MAA-LDL (Figure 12), and apoptotic Jurkat cells (data shown in the original publication III, Figure 6C), suggesting cross-reaction similar to that observed with human monoclonal Fab and human plasma antibodies.

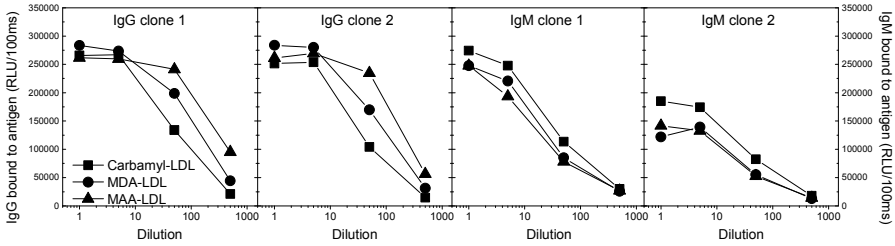

Fig. 12. IgG and IgM antibodies secreted by EBV-infected human B-cells bind to carbamyl-LDL, MDA-LDL and MAA-LDL. Media were collected and tested undiluted and with 1:5, 1:50 and 1:500 dilution. RLU = relative light unit.

5.7 Humoral immune response to carbamyl-LDL in LDLR$^{-/-}$ mice (I)

Mouse immunization study was carried out to investigate the humoral immune response to mouse carbamyl-LDL in LDLR$^{-/-}$ mice without any adjuvant, and also the effect of immunization on atherogenesis after high fat diet. Mice were immunized and fed with HFD, and the blood samples were taken as illustrated in Figure 5. IgG and IgM antibody binding to carbamyl-LDL, native LDL, CuOxLDL and MDA-LDL were tested from the plasma samples taken at the beginning of the study, after immunizations but before HFD, and at the end of the study.

The IgG titers to carbamyl-LDL increased during the study in mice immunized with mouse carbamyl-LDL (Figure 13A). IgG binding to carbamylated albumin was also tested, and shown to be similarly elevated in mice immunized with carbamyl-LDL (Figure 13B). Additionally, an increase in IgG binding to MDA-LDL was observed in carbamyl-LDL immunized mice (Figure 13 A). The IgM titers to carbamyl-LDL, but also to native LDL, OxLDL and MDA-LDL, were increased in all mice during the study (Figure 13 A), suggesting other influencing factors than immunization, such as ageing and HFD.

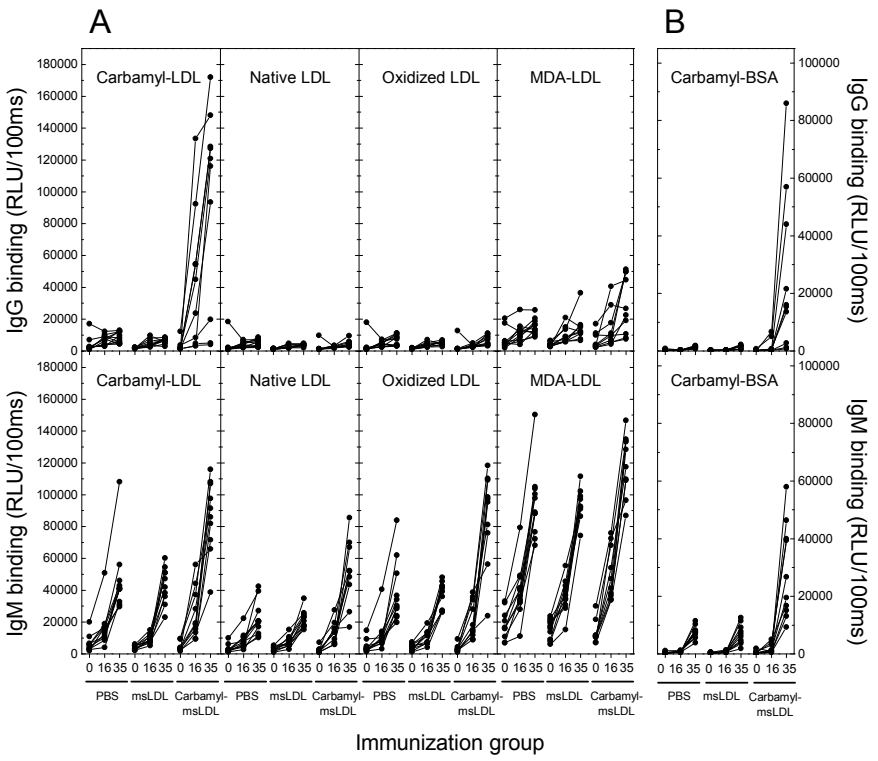

Fig. 13. Immune response in immunized LDLR$^{-/-}$ mice. Plasma IgG and IgM antibody levels to carbamyl-LDL, native LDL, oxidized LDL and MDA-LDL (A), and to carbamyl-BSA (B) in LDLR$^{-/-}$ mice immunized with PBS (n = 11), mouse LDL (msLDL, n = 10) or carbamylated mouse LDL (carbamyl-msLDL, n = 10). Plasma samples (1:1000) were measured at different times of the study: at the beginning of the study (0 weeks), after immunizations (16 weeks) and at the end of the study (35 weeks). Immunizations were carried out without adjuvants. RLU = relative light unit. Reprinted from the original publication I Figure 2 with permission from Mary Ann Liebert.

Total IgG and IgM concentrations in mouse plasma samples were determined and found to be increased during the study in all mice regardless of the immunization antigen (Figure 14).

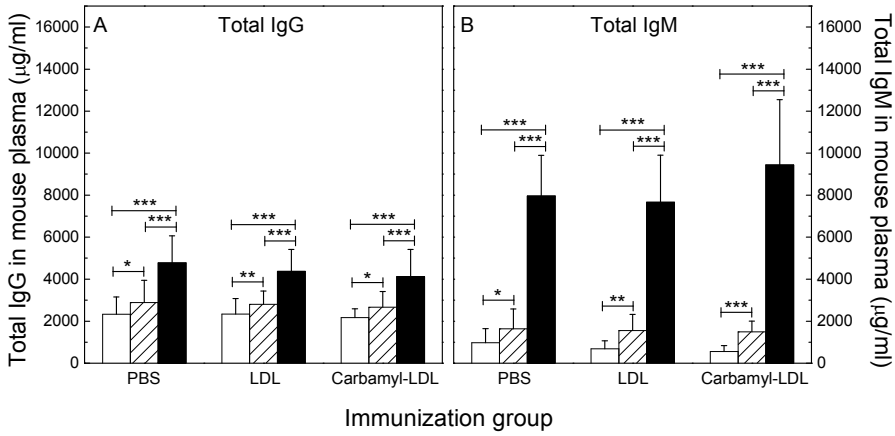

Fig. 14. Total antibody concentrations in LDLR$^{-/-}$ mice. Plasma total IgG (A) and IgM (B) concentrations (µg/ml) in samples taken at the beginning of the study (0 weeks, white columns), after immunizations (16 weeks, striped columns) and after high fat diet (35 weeks, black columns) in mice immunized with carbamyl-msLDL, msLDL or PBS. Reprinted from the original publication I Figure 5 with permission from Mary Ann Liebert.

Due to the increase in total antibody levels in all mice, the antigen-specific IgG and IgM antibody levels were normalized by the total antibody concentrations. Figure 15 illustrates that only increase in IgG level to carbamyl-LDL (Figure 15B) in carbamyl-LDL immunized mice remained statistically significant after the normalization.

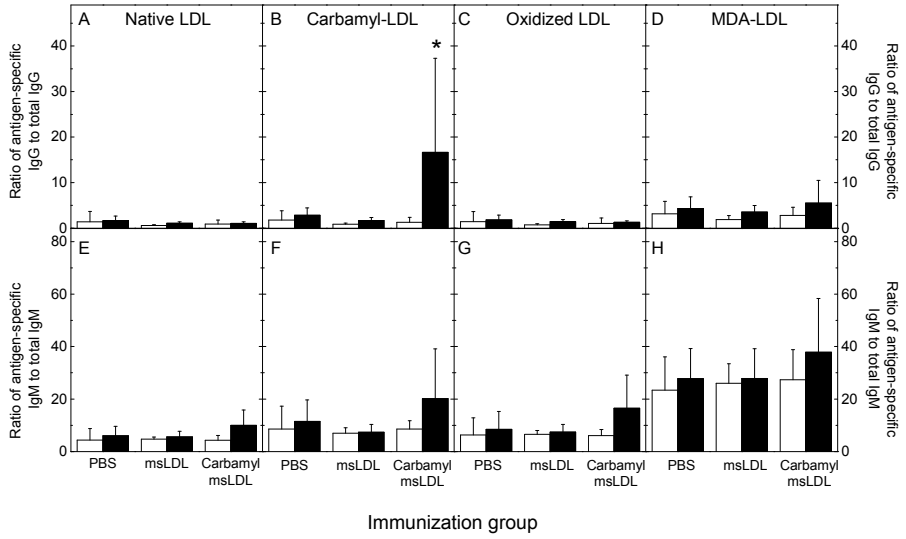

Fig. 15. Normalized plasma antibody levels in LDLR$^{-/-}$ mice. Antigen specific IgG and IgM levels (shown in Figure 13) were normalized by total IgG and IgM levels (shown in Figure 14) at the beginning of the study (0 weeks, white columns) and after high fat diet (35 weeks, black columns). Normalized plasma IgG (A-D) and IgM (E-H) levels to native LDL, carbamyl-LDL, copper-oxidized LDL and MDA-LDL. Triplicates for each mouse sample were measured and mean (SD) within a group is shown. Modified from the original publication I Figure 5 and reprinted with permission from Mary Ann Liebert.

The specificity of IgG and IgM immune responses in LDLR$^{-/-}$ mice were investigated in competitive liquid phase immunoassay. Soluble carbamyl-LDL, but also MDA-LDL, was able to compete for plasma IgG binding to immobilized carbamyl-LDL, whereas native LDL or OxLDL were not (Figure 16).

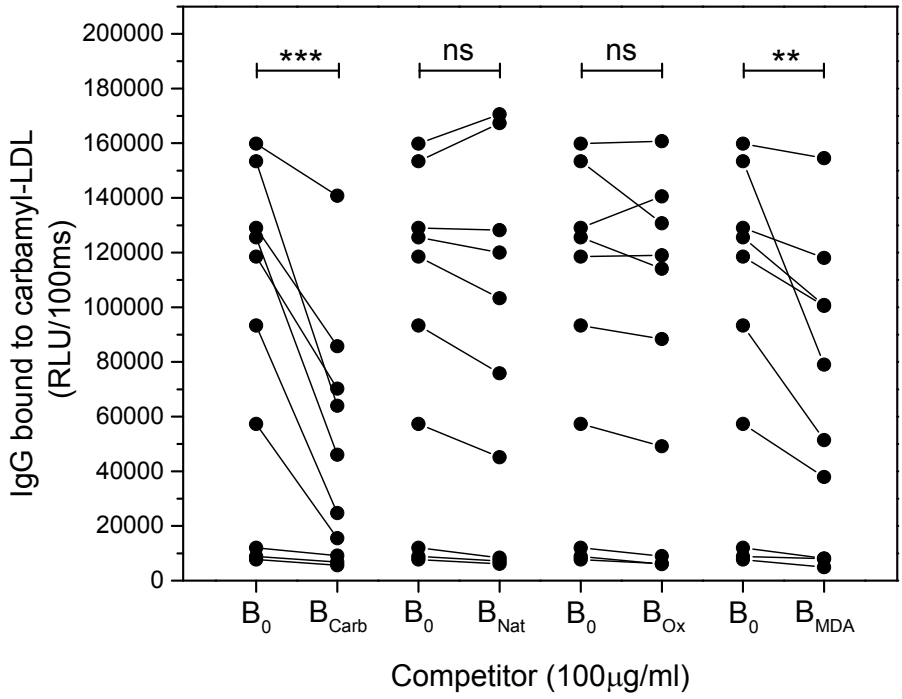

Fig. 16. Competitive immunoassay to test the specificity of mouse plasma (1:1000) IgG binding. Plasma IgG binding to immobilized carbamyl-LDL in the presence (100 µg/ml) or absence of competitors in all carbamyl-LDL immunized mice (n = 10). Carbamyl-LDL (B_{Carb}), native LDL (B_{Nat}), oxidized LDL (B_{Ox}) and MDA-LDL (B_{MDA}) were used as competitors. B_0 = binding without competitor. RLU = relative light units. Reprinted from the original publication I Figure 3 with permission from Mary Ann Liebert.

Antigen-specific IgM levels were elevated in all mice during the study. Therefore, the specificity of plasma IgM binding was tested in all mouse plasma samples, but differences were not observed between the competitors, or between the immunization groups. Statistical significance was reached when carbamyl-LDL, but also when native LDL, CuOxLDL and MDA-LDL were added as competitors. Decrease in IgM binding in competitive immunoassay was observed in all mice immunized with carbamyl-LDL, native LDL or PBS (data shown in the original publication I, Figure 4).

The presence of both carbamyl- and MDA epitopes on mouse plasma LDL particles was investigated. Carbamyl-epitopes were detected using rabbit

polyclonal antibody for homocitrulline (Turunen *et al.* 2010), and MDA-epitopes using mouse monoclonal IgM for MDA-LDL (Turunen *et al.* 2012). An increase in the amount of carbamyl-epitopes was observed in all mice during the study, but the highest levels were detected in mice immunized with carbamyl-LDL at the end of the study (Figure 17A). The MDA-epitopes were statistically increased during the study only in mice immunized with carbamyl-LDL (Figure 17 B).

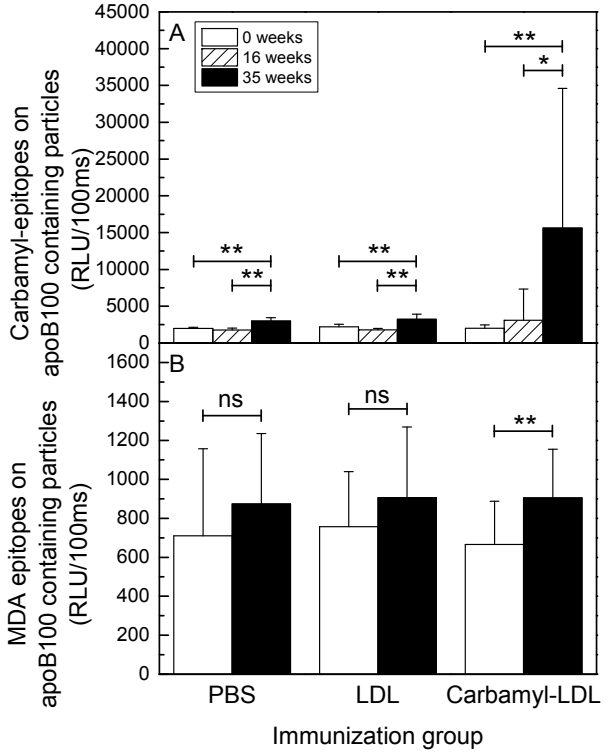

Fig. 17. Carbamyl- and MDA-epitopes in mouse plasma. Carbamyl-epitopes on apoB100 containing particles (A) and MDA epitopes on apoB100 containing particles (B) in mice immunized with carbamyl-LDL), native LDL or PBS. Carbamyl-epitopes were detected with rabbit IgG antibody specific for homocitrulline and MDA-epitopes with mouse IgM antibody specific for MDA-LDL. RLU = relative light units. Triplicates for each mouse sample were measured and mean (SD) within a group is shown. Reprinted from the original publication I Figure 7 with permission from Mary Ann Liebert.

At the end of the study, after immunization and high fat diet for 20 weeks, the mice were sacrificed and the amount of atherosclerosis was determined. Carbamyl-LDL immunization did not affect the atherosclerotic lesion area measured either in heart aortic-origin cross-section or *en face* aortas (Figure 18 A-B). However, the total number of atherosclerotic plaques (data not shown) and atherosclerotic plaques / μm^2 was significantly higher in mice immunized with PBS compared to mice immunized with carbamyl-LDL (Figure 18C). A representative *en face* aorta from each group is shown (Figure 18D-E).

Carbamyl-epitopes were detected from atherosclerotic lesions of the immunized mice using the rabbit polyclonal IgG recognizing homocitrulline in immunohistochemical staining. There were no quantitative differences in the amount of staining between the immunization groups (representative stained heart cross-sections shown in the original publication II, Figure 8F-H).

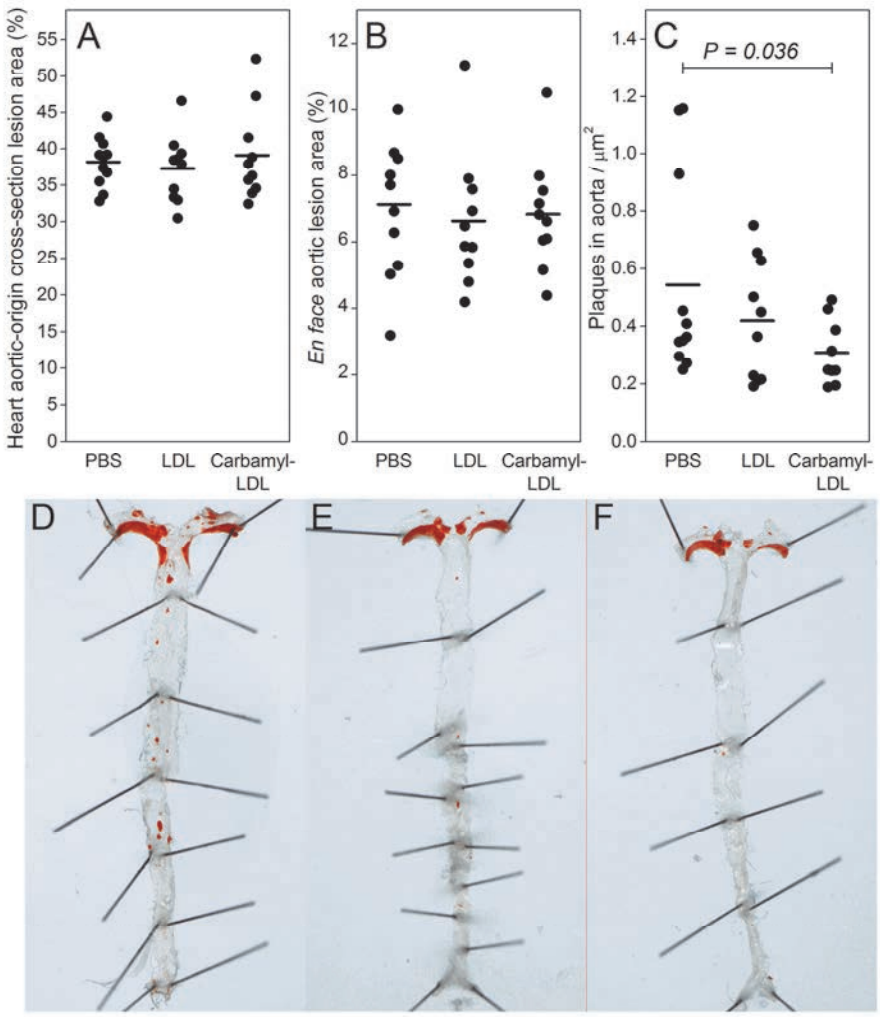

Fig. 18. Atherosclerosis in LDLR$^{-/-}$ mice immunized with carbamyl-LDL, native LDL or PBS. The lesion area in the cross-sections of aortic origin (A), the total plaque area in the *en face* mouse aortas (B) and number of plaques in aorta / µm^2 computed with MCID Core 7.0 analysis software (InterFocus Imaging Ltd). An aorta from one mouse immunized with PBS (D), LDL (E) or carbamyl-LDL (F). Modified from the original publication I Figure 8 and reprinted with permission from Mary AnnLiebert.

5.8 Antibody cross-reactivity with apoptotic cells (II, III)

Antibody binding to UV-irradiated Jurkat cells was investigated in this study by fluorescence microscopy and flow cytometry. Human monoclonal Fab106 was shown to bind UV-irradiated Jurkat cells undergoing apoptosis, as demonstrated in fluorescence microscopy (Figure 19).

Apoptotic cells were distinguished from non-apoptotic cells by propidium iodide (PI) or SYTOX AADvanced staining, and gated. Antibody binding to apoptotic and non-apoptotic cell populations was then separately analyzed. Fab106 bound to one third of the apoptotic cell population (Figure 19), but did not bind to non-apoptotic cells (Figure 19) (data also shown in the original publication II, Figure 5 and Supplemental Figure 1).

The binding specificity was investigated in competitive assay by the addition of carbamyl-, MDA- and MAA-modified proteins as competitors. The binding of Fab106 to apoptotic cells was decreased by 40% when carbamyl-LDL was added and by 74% when caramyl-BSA was added as competitor. MDA-LDL, MDA-BSA and MAA-BSA were able to decrease Fab106 binding to apoptotic cells by over 80%. Native LDL or native BSA did not compete for Fab106 binding to apoptotic cells (data shown in the original publication II, Figure 6).

IgG and IgM antibodies secreted by EBV-infected human B-cell clones were also tested for binding to apoptotic cells. Similarly to the monoclonal human Fab106, all four clones showed binding to apoptotic cells (data shown in the original publication III, Figure 6C).

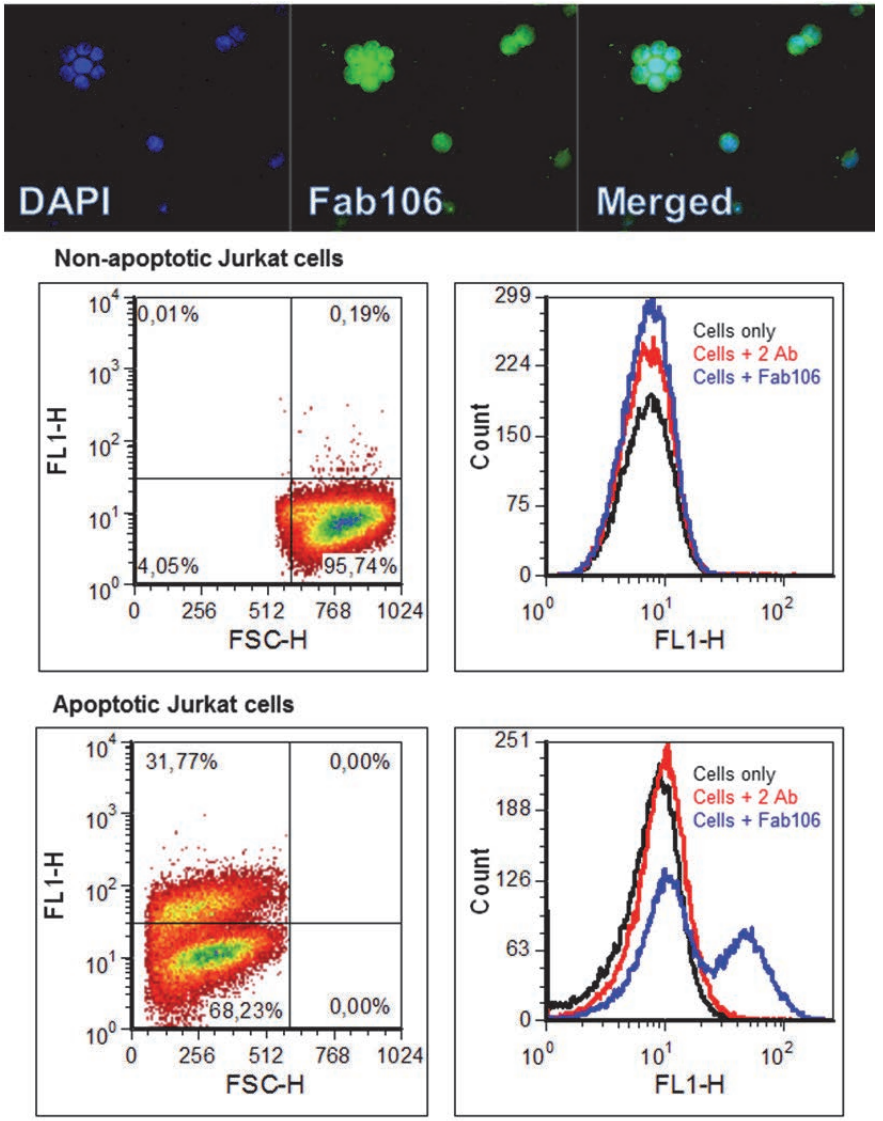

Fig. 19. Fab106 bound to UV-irradiated Jurkat cells undergoing apoptosis, demonstrated in immunofluorescence microscopy and flow cytometry. Fab106 bound 30% of apoptotic cell population and did not bind non-apoptotic viable cells, detected by flow cytometry. Modified from the original publication II Figure 5 and reprinted with permission from John Wiley & Sons.

Human plasma IgG and IgM antibody binding to apoptotic cells were analyzed similarly. Differences were observed in antibody binding to apoptotic cells between different human plasma samples (data shown in the original publication III, Figure 1). When the study groups were compared, IgG antibody binding to apoptotic cells was significantly higher in hemodialysis patients as compared to healthy controls (Figure 20A). However, there was no difference in IgM antibody binding between the groups (Figure 20B).

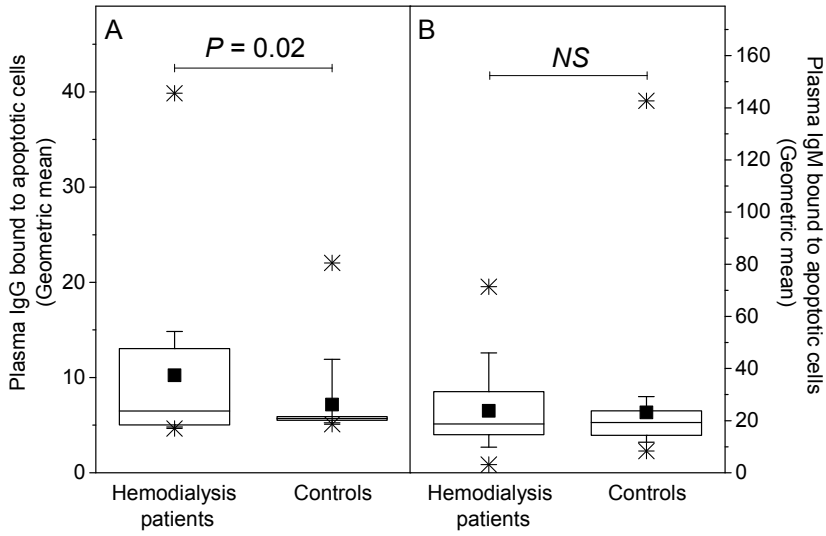

Fig. 20. Plasma IgG and IgM antibody binding to apoptotic cells in hemodialysis patients (n = 39) and healthy controls (n = 42) demonstrated by flow cytometry. Geometric mean values for apoptotic population that were bound by antibodies are shown. The box plots represent 25%, 50% and 75%, and the whiskers represent 10% and 90% distribution of values within the groups. The asterisks are minimum and maximum values and the squares are the mean values.

The total IgG and IgM antibody concentrations were similar between the study groups (data shown in the original publication III, Figure 2C-D), verifying that the difference observed in IgG to apoptotic cells was not due to the difference in total IgG levels. Competition assays were performed to test plasma IgG antibody specificity, and again, variation was seen between plasma samples of hemodialysis patients. Carbamylated proteins competed out plasma IgG binding to apoptotic cells significantly in some plasma samples, as demonstrated in flow

cytometry assay and fluorescence microscopy (data shown in the original publication III, Figure 5).

Also, mouse plasma samples taken at the end of the immunization study were tested for antibody binding to apoptotic cells. There was a statistically significant difference in IgG and IgM binding to apoptotic cells in mice immunized with carbamyl-LDL compared to PBS (Figure 21).

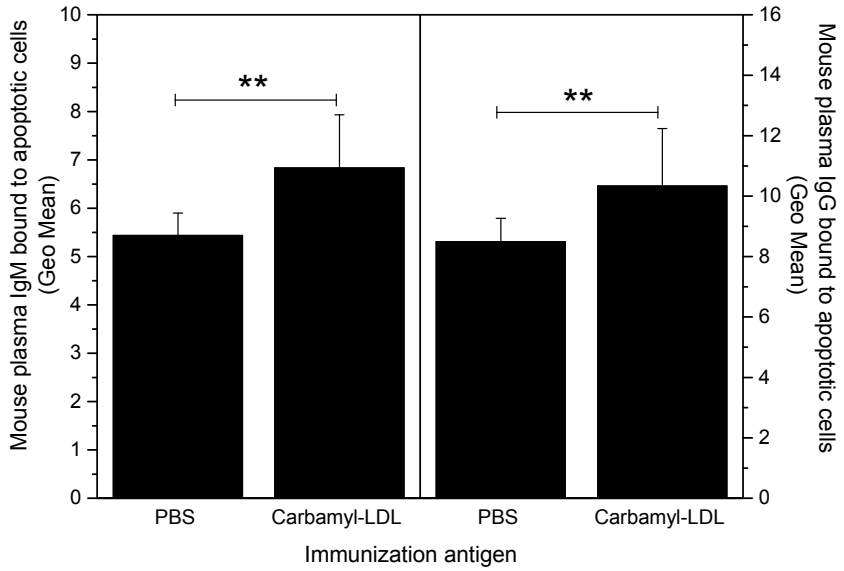

Fig. 21. Mouse plasma IgG and IgM binding to apoptotic Jurkat cells. Mice immunized with carbamyl-LDL had more IgM and IgG antibodies binding to apoptotic cell shown by flow cytometry. Geometric means (mean ± SD) for apoptotic population bound by antibodies are shown.

6 Discussion

Carbamylation is a post-translational modification playing important role in the context of uremia, smoking, inflammation and atherogenesis (Wang *et al.* 2007). Humoral immune responses induced by modified low-density lipoprotein are suggested to participate in atherogenesis (Ait-Oufella *et al.* 2010, Binder 2010, Lewis *et al.* 2009). This thesis investigated the presence, properties, and role of antibodies to carbamylated proteins in humans and in carbamyl-LDL immunized mice.

6.1 Humoral immune response to carbamyl-epitopes in humans

6.1.1 Plasma autoantibodies in humans

Autoantibodies to carbamylated (homocitrulline-containing) proteins have recently been demonstrated, for the first time, in humans. Anti-carbamyl-antibodies were measured in patients with rheumatoid arthritis, and IgG anti-carbamyl-antibodies were suggested to be predictive for a more severe course of the disease (Shi *et al.* 2011). At the beginning of this thesis project, autoantibodies to carbamylated proteins were not yet demonstrated in humans. Hemodialysis patients were selected because they have high plasma urea levels and increased carbamylation of plasma proteins. In the current study (I) IgG and IgM antibodies to carbamylated LDL and carbamylated protein were detected in human plasma, even in healthy subjects. Plasma IgG levels to carbamyl-modified proteins were shown to be elevated in uremic patients and in smoking subjects, both known to have increased carbamylative burden *in vivo*. The antibody levels were the highest among smokers with elevated plasma urea levels, and the lowest in non-smokers with normal urea levels. This suggests the involvement of both carbamylation routes: nonenzymatic urea-dependent and MPO-catalyzed carbamylation. Similar difference was not observed in IgM antibody levels. The absence of IgM antibody response suggests that the immune response to carbamyl-epitopes is secondary IgG, rather than primary IgM. The patients with CKD may have lower total immunoglobulin levels compared to healthy subjects and humoral immune response induced by some pathogens, such as hepatitis virus B in vaccination, has been shown to be diminished (Chaves *et al.* 2011, Duranti & Duranti 2011, Neu 2012). The reduced antibody production in patients

with end-stage renal disease is caused by impairment in T-cell activation, and reduced number of B-lymphocytes and antigen presenting dendritic cells (Agrawal *et al.* 2010, Pahl *et al.* 2010). Therefore, the increased IgG level to carbamylated proteins are not likely to result from other kidney disease specific factors, or medications, but rather from the increased carbamylation.

Protein carbamylation was suggested to be a marker predicting increased risk for coronary artery disease, and the amount of systemic ε-carbamyl-lysine to be used as a quantitative index of cardiovascular risk (Wang *et al.* 2007) and recently shown to predict mortality in patients with ESRD (Berg *et al.* 2013, Koeth *et al.* 2013). The homocitrulline measurement from plasma samples requires special equipment, such as HPLC with online tandem mass spectrometry (Wang *et al.* 2007). Based on the findings in this study, plasma IgG antibodies to carbamyl-adducts may indicate elevated homocitrulline levels, and should be further investigated as possible markers of cardiovascular disease related to increased carbamylation.

Cross-reactivity of human plasma antibodies recognizing carbamyl-epitopes and oxidation-specific epitopes

Previously published data concerning plasma antibodies to carbamyl-LDL or carbamylated proteins in humans is limited, and autoantibodies have been only recently described. This thesis study (II) demonstrates that antibodies to carbamyl-LDL and carbamylated proteins are associated with antibodies to oxidation-specific epitopes in human plasma samples. The association was seen in IgM and IgG antibody isotypes, suggesting the role *in vivo* could be both natural and adaptive.

A different cross-reaction related to anti-homocitrulline (anti-carbamyl) and anti-citrulline antibodies, both present in the plasma of rheumatoid arthritis patients, has been reported (Shi *et al.* 2011, Shi *et al.* 2013). Citrulline and homocitrulline (carbamyl-lysine) share very similar structures, but homocitrulline is longer by one methylene (-CH_2) group (Trouw *et al.* 2013). Anti-citrullinated protein antibodies (ACPA) are used in the prognosis and diagnosis of rheumatoid arthritis. Patients who tested negative for ACPA were shown to have more IgG and IgA antibodies recognizing carbamylated proteins, and predicting more severe disease (Shi *et al.* 2011). Anti-carbamyl antibodies also predicted the onset of reumathoid arthritis in arthralgia patients (Shi *et al.* 2013). Anti-carbamyl antibodies have also been detected in some patients with juvenile idiopathic

arthritis (Muller *et al.* 2013). Citrulline-epitopes have been demonstrated in atherosclerotic lesions and suggested to be a possible target for anti-citrullinated protein antibodies, and the resulting immune complexes might promote atherogenesis in the patients with rheumatoid arthritis.

Traditional risk factors do not account for the higher incidence of atherosclerosis observed in the patients with rheumatoid arthritis and chronic kidney disease (Apostolov *et al.* 2012, del Rincon *et al.* 2001, Lindner *et al.* 1974, Solomon *et al.* 2006). A question arises, whether some shared factors influence the atherogenesis in these two chronic diseases. One common factor seems to be the antibodies recognizing carbamylated proteins. The data regarding antibody cross-reactivity between carbamyl- and oxidation-specific epitopes represented in this thesis suggest a possible role in the atherogenesis in the patients with CKD.

Human plasma antibodies bound to apoptotic cells

Apoptosis in blood mononuclear cells is increased in patients receiving hemodialysis treatment (Atamaniuk *et al.* 2006, Galli *et al.* 2003, Heidenreich *et al.* 1996, Jaber *et al.* 2001, Martin-Malo *et al.* 2000, Matsumoto *et al.* 1995, Matsumoto *et al.* 1995). Both hemodialysis and uremia can explain the enhanced apoptosis (Bhaskaran *et al.* 2002, Martin-Malo *et al.* 2000, Ranjan *et al.* 2002). Hemodialysis patients are shown to have serum factors that promote T-lymphocyte apoptosis time- and dose-dependently (Bhaskaran *et al.* 2002), and susceptibility of CD4+ T-cells to activation-induced apoptosis (Ankersmit *et al.* 2001). Enhanced oxidative stress, and accumulation of oxidized lipids, proteins and nucleic acids are suggested as possible contributing factor (Bordoni *et al.* 2006). Oxidized cardiolipin contributes to apoptosis, and low plasma IgM antibodies to oxidized cardiolipin have recently been shown to associate with increased death in hemodialysis patients (Frostegård *et al.* 2013).

Plasma antibodies to apoptotic cells, both IgG and IgM, have been previously described in humans (Ciurana & Hack 2006, Diepenhorst *et al.* 2012, Vay *et al.* 2006, Zwart *et al.* 2004). IgG antibodies to apoptotic cells have been demonstrated e.g. in patients with alcoholic liver disease and suggested to target oxidized phosphatidylserine and participate in hepatocyte apoptosis (Vay *et al.* 2006). Isolated plasma IgG have been shown to opsonize apoptotic cells, and inhibit the uptake by macrophage Fcγ-receptors in patients with systemic lupus erythematosus (SLE), proposing a functional role (Reefman *et al.* 2007). SLE patients have enhanced LDL oxidation and increased antibody levels to OxLDL,

which are associated with arterial disease and renal manifestations (Frostegård *et al.* 2005). Antibodies against apoptotic cells have not been previously described in hemodialysis patients. This study (III) showed an increased amount of human plasma IgG antibodies binding to apoptotic Jurkat cells in hemodialysis patients compared to healthy controls. Hemodialysis patients were also shown to have more IgG antibodies to carbamyl-epitopes (I, III) and to oxidized LDL and oxidation-specific MAA-epitope (III). However, the amount of IgG binding to apoptotic cells did not correlate with the IgG antibodies to carbamyl-LDL, OxLDL, or MAA-LDL. IgG binding to apoptotic cells did not associate with the diagnosis for kidney failure, and the patients having the highest binding to apoptotic cells had different causes for kidney failure and dialysis.

IgM antibodies to apoptotic cells have been investigated in several studies (Ciurana & Hack 2006, Diepenhorst *et al.* 2012, Zwart *et al.* 2004), and have been suggested to recognize mostly PC-epitopes (Ciurana & Hack 2006, Kim *et al.* 2002) present on OxLDL, and on the surface of apoptotic cells (Chang *et al.* 1999). IgM antibodies to OxLDL have proposed to be atheroprotective in humans (Karvonen *et al.* 2003, Tsimikas *et al.* 2012). IgM, with PC-specificity, have been suggested to initiate the complement activation during apoptosis (Kim *et al.* 2002, Zwart *et al.* 2004). Low IgM levels to PC are associated with increased mortality in dialysis patients (Carrero *et al.* 2009). IgM antibodies act in the clearance of apoptotic cells *in vivo*, and this role may be disturbed in dialysis patients. Inefficient clearance of apoptotic cells might lead to dysfunction in normal homeostasis, and the generation of humoral IgG response as seen in this study (III).

Role of carbamylation and autoantibodies in atherogenesis

Carbamyl-adducts are formed *in vivo* by urea-dependent or MPO-catalyzed carbamylation (Wang *et al.* 2007), in a reaction with cyanate (Stark *et al.* 1960, Stark 1965). LDL carbamylation has long been suggested to contribute to the high incidence of atherosclerosis in patients with elevated urea levels (Apostolov *et al.* 2007, Gonen *et al.* 1985, Hörkkö *et al.* 1992, Hörkkö *et al.* 1994). Two separate studies have recently shown that protein carbamylation predicts mortality in patients with ESRD (Berg *et al.* 2013, Koeth *et al.* 2013). Protein-bound homocitrulline (PBHCit) (Koeth *et al.* 2013) and carbamyl-albumin (Berg *et al.* 2013) were measured in these studies by mass spectrometry, and shown to associate with mortality after follow-up. Carbamyl-adducts are found in

atherosclerotic lesions, where they colocalize with MPO known to catalyze carbamylation (Wang *et al.* 2007). Oxidation-specific MDA- and MAA-adducts are also found in lesions.

IgG antibody levels to carbamyl-epitopes were shown to be elevated in hemodialysis patients (I), and the difference was still significant after normalization with total IgG concentration (III). Hemodialysis patients also demonstrated elevated relative IgG levels to oxidation-specific epitopes, OxLDL and MAA-LDL (III). Antibodies to carbamyl-epitopes associated with antibodies to oxidation-specific epitopes in hemodialysis patients (Table 5) and healthy controls (II).

Even though the role of antibodies in the development of atherosclerosis has been widely studied, the data still remains inconclusive. Several potential target antigens, different detection methods, and the lack of standardized method for measurement of antigen-specific antibody levels have hampered the comparison of the seemingly similar studies. Furthermore, different antibody isotypes have been investigated. Some of the first studies in the early nineties showed that IgG antibody levels to OxLDL would predict the progression of atherosclerosis (Salonen *et al.* 1992). In patients with chronic kidney disease and uremia, the antibody ratio to OxLDL and native LDL were shown to be increased (Bellazzi *et al.* 1993, Maggi *et al.* 1994a, Maggi *et al.* 1994b, Maggi *et al.* 1994c). The antibodies to OxLDL can be markers of oxidative stress during the development of atherosclerosis. On the contrary, an inverse association between coronary artery disease and IgG antibodies to OxLDL has been observed (Lopes-Virella *et al.* 1999). This was proposed to result from IgG immune complex formation that would affect the measurement of free IgG antibodies.

The studies detecting IgM type of antibodies to OxLDL in humans have often shown correlation with decreased atherosclerosis (Karvonen *et al.* 2003, Su *et al.* 2006, Tsimikas *et al.* 2012). IgM antibodies to oxidation-specific epitopes cloned from mice have shown to possess atheroprotective properties. These natural antibodies for example block the OxLDL uptake through scavenger receptors in macrophages (Hörkkö *et al.* 1999, Tuominen *et al.* 2006).

6.1.2 Human monoclonal Fab antibody to carbamyl-epitopes

Human monoclonal Fab antibodies cloned in this study (II) originated from healthy individuals. The selection was made based on the binding to carbamyl-LDL and the Fab antibodies shared high homology with germline genes. One Fab

clone IFP-02_106, also called Fab106, was studied in detail and observed to have cross-reactive properties. Fab106 bound to carbamyl-epitopes and also oxidation-specific MDA-adducts, and apoptotic cells. These data imply the natural origin of Fab106. Fab106 is an antibody fragment lacking the Fc-domain and it has been constructed by amplification of only the antibody variable regions from donor lymphocytes. Isotype-determining constant regions are amplified from the templates cloned into the vectors pCom3XTT and pComb3Xλ, and thus the isotype origin of the antibody is not known.

The isotype of natural antibodies is usually IgM and their sequence is very similar, or identical to germline genes. Natural IgM are polyreactive and able to bind a variety of unencountered antigens, but have low affinity (Binder 2010, Manson *et al.* 2005). Primary low-affinity IgM immune response is followed by the secondary high-affinity IgG with a specific target. Natural IgM antibodies have very important role in maintaining the cellular homeostasis and participating in the clearance of dying cells undergoing apoptosis (Kaveri *et al.* 2012). IgM antibodies have shown to bind late apoptotic, PI-positive human Jurkat cells via antibody's Fab domain, whereas Fab digested from IgG antibodies did not (Kim *et al.* 2002).

One of the original aims in this study was to clone carbamyl-specific Fab antibody. Fab106 was selected, based on the binding to carbamyl-LDL, but was found to bind also to other carbamyl-adducted proteins, thus suggesting that it was specific for the carbamyl-epitope and not the carrier protein. In later studies, a specific cross-reaction between carbamyl- and MDA/MAA-adducts was observed with human monoclonal Fab106 (II). Fab106 was observed to cross-react with MDA- and MAA-epitopes in LDL and albumin, but no binding to PC-epitope was evidenced. This was surprising, since the modifications in question seemed to be very distinctive. LDL accumulates in the arterial wall and is exposed to oxidation. Oxidation-specific epitopes are formed in the presence of highly reactive end-products of lipid peroxidation that modify proteins and lipids (Hörkkö *et al.* 2000). An abundant dialdehyde generated during lipid peroxidation is malondialdehyde (MDA), and it eagerly reacts with primary amines resulting in MDA-adducts (Esterbauer *et al.* 1991). Another oxidation product, acetaldehyde, forms malondialdehyde acetaldehyde (MAA)–adducts in the presence of malondialdehyde (Tuma *et al.* 1996, Tuma *et al.* 2001). Copper oxidized LDL is commonly used in laboratory experiments as a model of LDL oxidation. It contains a variety of different epitopes which include MDA-adducts. The major epitope in the copper oxidized LDL is phosphocholine (PC)-head groups of

oxidized phospholipids, such as 1-palmitoyl-2-(5'-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC) (Palinski *et al.* 1996). These oxidation-specific epitopes are also present in apoptotic cells (Chang *et al.* 1999) and found in atherosclerotic lesions (Palinski *et al.* 1996), and suggested to participate in immune modulation of atherosclerosis. Antibodies recognizing different epitopes found in OxLDL and apoptotic cell may have various, and even opposite effects in atherogenesis. The monoclonal Fab106 antibody also bound to epitopes found in atherosclerotic lesion and apoptotic cells. However, the true epitope recognized by Fab106 in apoptotic cells still remains unknown.

Monoclonal human Fab antibodies have previously been cloned against MDA-LDL (Shaw *et al.* 2001) and oxidized LDL (Jeon *et al.* 2007). In both cases the phage display libraries were constructed from patients with atherosclerotic disease. High germline gene homology was revealed in sequence analysis of these antibodies. They were able to block OxLDL uptake by macrophages and bind to atherosclerotic lesions. Fab106 showed similar functional properties that previously described Fabs to OxLDL, even though it originates from healthy humans against different antigen. Fab106 was homologous to germline genes, recognized epitopes in atherosclerotic plaque, and inhibited the uptake of modified LDL in macrophages, which is a necessary step in the formation of foam cells initiating atherosclerotic lesion. The Fab106 cloned in this study differs from the previously published Fabs in its unique feature not described earlier; cross-reaction between carbamylated and MDA-/MAA-modified oxidation-specific epitopes but not with PC-epitopes. The cross-reactivity between carbamyl- and malondialdehyde-adduct was also characteristic to polyclonal repertoire of human plasma antibodies, supporting the *in vivo* existence of natural antibodies with features similar to the Fab106 described in this study.

6.1.3 Antibodies secreted by human EBV-infected B-cells

The preliminary data obtained from human B-cells secreting antibodies *in vitro* after immortalization with EBV (III) supports the findings obtained with human plasma antibodies (I, II) and human monoclonal Fab106 antibodies (II) in this study. IgG and IgM antibodies secreted by EBV-immortalized B-cells demonstrated cross-reactive binding to carbamyl-LDL, MDA-LDL and MAA-LDL, and bound to epitopes on apoptotic cells. The EBV-immortalization of B-cells allows production and purification of human monoclonal antibodies

identical to those existing *in vivo*. Monoclonal Fab106 antibody cloned and investigated in this study (II) is assembled *in vitro*, and may not have existed *in vivo* as such. Both heavy and light chain variable regions were amplified from peripheral blood lymphocytes, but RNAs from four individuals were mixed and the pairing of heavy and light chains was random. Plasma antibodies to carbamyl-epitopes were detected (I), shown to associate with oxidation-specific epitopes (II), and to bind to apoptotic cells (III). However, in case of antibody repertoire found in plasma samples, it is not certain if the antibodies investigated in each of the assays are the same. The data obtained from antibodies secreted by EBV-infected B-cell clones (III) strongly propose the presence of cross-reactive antibodies *in vivo*.

6.2 The effect of carbamyl-LDL immunization in LDLR$^{-/-}$ mice

The mouse immunization study included in this thesis was designed to investigate the humoral immune response triggered by carbamyl-LDL immunization in LDLR$^{-/-}$ mice, and the effect of carbamyl-LDL immunization in the development of atherosclerosis in these mice on high fat diet compared to mice immunized with native LDL or PBS. Previously, the immune response to homologous carbamyl-LDL immunization has been studied in guinea pigs (Steinbrecher *et al.* 1984a), but the effect on atherogenesis has not been addressed.

6.2.1 Humoral immune response

Mouse LDL modified *in vitro* by carbamylation without adjuvant triggered adaptive humoral immune response in LDLR$^{-/-}$ mice; IgG antibodies to carbamyl-LDL were induced in mice immunized with carbamyl-LDL. This finding is consistent with the previous study in guinea-pigs (Steinbrecher *et al.* 1984a). The functional role of humoral immune response was further investigated. Mouse plasma IgG antibodies also recognized carbamyl-epitopes in albumin, indicating carbamyl-specific antibody response. IgG antibodies to native LDL or oxidized LDL were not detected, but carbamyl-LDL immunization was shown to induce minimal specific IgG antibody response to MDA-LDL (I). Elevated MDA levels (Boaz *et al.* 1999, Tamer *et al.* 2002) and MDA-LDL (Viigimaa *et al.* 2010) are shown in humans with enhanced atherosclerosis. MDA-modified proteins are shown to be immunogenic and immunization with MDA-LDL is shown to protect LDLR$^{-/-}$ mice from atherosclerosis (Freigang *et al.* 1998).

Specificity of IgG antibody response was tested in liquid phase competitive immunoassay. Soluble MDA-LDL competed for plasma IgG binding to immobilized carbamyl-LDL; approximately 25% of the IgG binding was competed, thus suggesting the existence of cross-reactive epitopes between carbamyl-LDL and MDA-LDL generated after carbamyl-LDL immunization. OxLDL was not able to compete with IgG binding to carbamyl-LDL, nor was native LDL. It is possible that after injection into the mouse, carbamyl-LDL is internalized by the antigen presenting cells *in vivo*, and the lipid moiety of LDL undergoes oxidation creating MDA-epitopes. This explanation was further supported by the increase in MDA-LDL levels in the plasma of $LDLR^{-/-}$ mice immunized with carbamyl-LDL, but not in mice immunized with native LDL or PBS. The IgG antibody response to MDA-LDL was not detected either in the control mice immunized with native LDL or PBS.

IgM antibody response observed in the immunized mice was different from the IgG response. IgM antibodies were elevated in all mice at the end of the study, against all antigens tested, including carbamyl-LDL, MDA-LDL, OxLDL and even native LDL. The fact that IgM response was triggered also in the mice injected with PBS buffer implies other causes than immunization, such as high fat diet. Also, the total IgM levels were elevated in all mice during the study, and there were no differences between the groups immunized with carbamyl-LDL, native LDL or PBS. The rise in total IgM levels is consistent with the other studies. Mice immunized with oxidized LDL (Pedersen *et al.* 2010) and MDA-LDL (Turunen *et al.* 2014) had elevated total IgM levels after high fat diet. Natural mouse antibodies are preferably IgM isotype and are suggested to target oxidation-specific epitopes *in vivo* (Binder 2010). IgM response was unlikely to influence the atherosclerosis results.

6.2.2 Effect of carbamyl-LDL immunization on atherosclerosis

Several earlier studies have shown that immunization with various modified LDL provides atheroprotection in animal models (Ameli *et al.* 1996, Freigang *et al.* 1998, Nilsson *et al.* 1997, Palinski *et al.* 1995, Zhou *et al.* 2001). Specific adaptive IgG immune response to carbamyl-epitope was observed in the mice immunized with carbamyl-LDL. When designing the immunization study, the hypothesis was that humoral immune response triggered by the immunization would affect, for example, the uptake of modified LDL through macrophage scavenger receptors, and influence the atherogenesis in these mice. Humoral

immune system was thought to participate via carbamyl-epitope itself, or through oxidation-specific epitopes formed from oxidation of PUFA containing lipids. In this study (II), the immunization of $LDLR^{-/-}$ mice with homologous carbamyl-LDL was not able to influence the development of total atherosclerotic lesion area in *en face* analysis of the whole aorta, or the heart aortic-origin cross-sections. However, a statistically significant difference was seen in the number of plaques in *en face* aortas. Mice immunized with PBS had more individual plaques / μm^2 than mice immunized with carbamyl-LDL. The rate of formation of new atherosclerotic plaques is shown to be a predictor of cardiovascular events in patients with ESRD, suggesting that monitoring plaque formation rather than intima-media thickness may be useful for assessment of cardiovascular risk (Benedetto *et al.* 2008).

Adjuvants are commonly used in the immunization studies to boost the immune response in animals. Many of the animal studies have used oil-water emulsion of antigen mixed with Freund´s adjuvant in investigating the role of modified LDL in the development of atherosclerosis (Freigang *et al.* 1998, Palinski *et al.* 1995, Zhou *et al.* 2001). However, Freund's adjuvant has been shown to ameliorate the disease in atherosclerotic $apoE^{-/-}$ mouse model (Khallou-Laschet *et al.* 2006). Elevated IgM antibody level to MDA-LDL after immunization with the Freund´s adjuvant was suggested to contribute to the atheroprotective effect. On the contrary, Freund´s adjuvant has been shown to enhance atherosclerotic lesion development in New Zealand White (NZW) rabbits (Xu *et al.* 1992). To avoid any interference in the development of atherosclerosis in $LDLR^{-/-}$ mice, the use of Freund´s or any other adjuvant was omitted in this immunization study (I). Homologous LDL carbamylated *in vitro* was shown to be immunogenic in mice and capable of inducing adaptive IgG immune response on its own. This is consistent with the earlier small immunization study in guinea pigs (Steinbrecher *et al.* 1984a). The comparison of the immunization studies, investigating the functional role in atherogenesis, is challenging for several reasons. The potential influence of adjuvant has not always been considered, and the use of different strains or even different animal species has complicated the comparison. Also, many different modifications in LDL have been used.

Carbamyl-LDL has several atherogenic properties. It is able to promote vascular smooth muscle cell proliferation and monocyte adhesion by ICAM-1 and VCAM-1 expression (Asci *et al.* 2008), which may contribute to the increased atherosclerosis in patients with chronic kidney disease. In addition, carbamylated

LDL induces oxidative stress and accelerates senescence in human endothelial progenitor cells (Carracedo *et al.* 2011), and these may have a role in enhanced atherogenesis, as well. Carbamyl-LDL is recognized by LOX-1 and CD36 scavenger receptors, whereas the transcytosis involves SR-A1, SREC-1, but not LOX-1 receptors (Apostolov *et al.* 2009). HDL can also be modified by carbamylation, and was shown to be the major posttranslational modification and affecting the atheroprotective properties of HDL (Holzer *et al.* 2011). Cyanate, rather than carbamylated lipoproteins, has been proposed to cause induction of ICAM-1 expression in human coronary artery endothelial cells and also after oral administration in endothelial cells in mouse aorta (El-Gamal *et al.* 2012).

In this study (I) the amount of carbamylated lysine-residues were measured in apoB containing lipoproteins in $LDLR^{-/-}$ mice. Polyclonal IgG purified from sera of NZW rabbit immunized with synthetic homocitrullinated peptide (CEKAHDGGRYHomocitA) was used in the assay (Turunen *et al.* 2010). $LDLR^{-/-}$ mice on regular chow diet had comparable amounts of carbamyl-LDL with C57BL/6 mice. When $LDLR^{-/-}$ mice were fed HFD, the amount of carbamyl-LDL increased. This may result from the increase in plasma urea levels in $LDLR^{-/-}$ mice during HFD. The amount of carbamyl-LDL in plasma was higher in mice immunized with carbamyl-LDL compared to control groups. This might be explained by the increased plasma MPO activity observed in the carbamyl-LDL immunized mice, and involvement of both carbamylation routes. The amount of carbamyl-LDL and IgG antibodies to carbamyl-epitopes in mouse plasma did not associate with each other, thus immunocomplexes are not likely to have a mechanistic role. Carbamyl-epitopes have previously been demonstrated in human atherosclerotic lesion (Wang *et al.* 2007). In this study (I) carbamyl-adducts were also detected in atherosclerotic plaques in $LDLR^{-/-}$ mice. The differences between immunization groups were not observed in immunohistochemical stainings, probably due to the fact that all mice had atherosclerosis and elevated urea levels, regardless of the immunization antigen.

6.3 Remaining questions

6.3.1 Study limitations and methodological considerations

Human plasma samples from dialysis patients and healthy controls were used in this study. Patient samples were collected from volunteers recruited from the

Dialysis Unit of Oulu University Hospital. Control samples were collected from healthy volunteers at the Clinical Research Center of Oulu University Hospital. The dialysis patients were considerably older compared to the controls, aged 59.2 ± 17.8 and 36 ± 8.8 years, respectively. In addition, sex distribution was uneven; there were 11 females and 28 males in the dialysis patient group and 26 females and 16 males in the control group. The controls were mostly staff members working at the Clinical Research Center, as defined by the Ethics Committee approval obtained for this study, and age- and sex-matched subjects were not available. Humoral immune response is likely to be suppressed in older people (Frasca & Blomberg 2011, Frasca *et al.* 2011), and also in patients with chronic kidney disease (Cohen & Horl 2012). The total IgG and total IgM levels were slightly lower in hemodialysis patients but there was not a statistically significant difference between the groups. Therefore, the difference in IgG antibody levels to carbamylated proteins between hemodialysis patients and controls is not likely to result from uneven distribution of age and sex.

LDL modified *in vitro* was used in this study. The LDL was isolated from human plasma, or from mouse plasma in immunization study, in our laboratory. Antioxidants, BHT and EDTA, were added to the freshly isolated LDL to prevent oxidation. However, it is impossible to totally avoid small degree of spontaneous oxidation in LDL. LDL is oxidized already *in vivo*, and may be oxidized further during isolation and storage. We have tested the native and modified LDL preparations for the absence of PC-epitopes with a specific monoclonal antibody (EO6), the absence of thiobarbituric reactive substances (TBARS), and analyzed the degree of modifications in lysine residues (TNBS). When the cross-reaction between carbamyl-epitopes and MDA-/MAA-adducts was observed, unintended carbamylation in MDA- and MAA-modifications was first suspected. To make sure that MDA- and MAA modified antigens are not carbamylated, they were analyzed with HPLC for detection of homocitrulline (carbamyl-lysine). Homocitrulline was detected only in carbamyl-LDL and carbamyl-BSA, not in the MDA- and MAA-modified antigens or native proteins. For easy comparison of the antigens, native LDL was analyzed in parallel with carbamyl-LDL in most assays. Also, albumin was modified and used in some of the assays, because it is a much smaller and simpler molecule than LDL, with 59 lysine residues to be modified. The use of BSA instead of LDL allows the investigation of the modified epitope, and the huge carrier, LDL, is not affecting the results.

Fab phage display library was constructed from healthy humans to obtain an antibody with binding specificity existing normally *in vivo*. Only one clone,

Fab106, was selected for large-scale production and detailed studies in this thesis. Preliminary screening of antibody-bearing phage particles was performed on hundreds of single clones, and small-scale production was carried out for dozens of them. After several experiments performed with Fab106, I have debated that more than one clone should have been chosen. More clones can still be selected, produced, and tested from the phage display library. Also, what was the isotype of the antibody with Fab106 binding specificity originally *in vivo*? Due to the cloning method used in the construction of the phage display library, the original isotype cannot be determined.

The $LDLR^{-/-}$ mice used in this study developed atherosclerosis and had elevated urea levels at the end of the study, but they did not have chronic kidney disease. Immunization of these mice with carbamyl-LDL once a month was not able to induce humoral antibody response that would have affected the atherogenesis, neither protecting nor promoting. The carbamyl-LDL could have been administered more often, e.g. using small automatic pump inserted surgically in the mouse, and thus better mimicking the constant exposure to carbamyl-LDL in chronic kidney disease. In addition, mouse model of uremia, e.g. nephrectomized mice, could have been used to study the immune responses to carbamyl-epitopes. Atherosclerosis has previously shown to be enhanced in nephrectomized mice, but antibody responses to carbamyl-epitopes have not been assessed in detail, as in this thesis study.

6.3.2 Future goals

The aim of this study was to investigate the humoral immune response to carbamyl-epitopes and clone human antibodies to carbamylated proteins. Several Fab antibody clones have been cloned and tested during this thesis project, but none of the characterized clones so far have been specific only to carbamyl-epitope; a cross-reaction with MDA- and MAA-adducts, and also with apoptotic cells, is recurrently observed.

One future aim is to further study the cross-reactive humoral immune response detected, and to investigate whether there is a common epitope found, for example on bacteria, that would bind antibodies generated for both carbamyl- and malondialdehyde-adducts. Furthermore, the true epitope in apoptotic cells targeted by the plasma antibodies and monoclonal Fab106 investigated in this thesis remains unrevealed, and its identification might give new insight into their role in atherosclerosis.

Another future goal is to clone solely carbamyl-specific antibodies, and several techniques are readily available. The traditional hybridoma technique allows easy cloning of mouse monoclonal antibodies. Mostly IgM type antibodies are obtained, but also IgG antibodies have been successfully cloned in our laboratory. IgGs are currently being cloned from non-immunized $LDLR^{-/-}$ mice fed with HFD for extended time period. These mouse monoclonal antibodies could be useful in research and in diagnostic purposes. On the other hand, phage display technique or EBV-immortalization of B-cell would provide fully human antibodies, and the usefulness would then be expanded also to therapeutic applications.

It would be tempting to further investigate the role of antibodies in the context of chronic kidney disease, uremia, and atherosclerosis. The induction of anti-carbamyl-antibodies is evident after immunization with carbamyl-LDL, similarly to the immunization studied with oxidation-specific epitopes. Mouse models for chronic kidney disease have been created, and they have shown to have increased atherosclerosis. Would it be possible, somehow, to induce atheroprotective antibody response in such mouse model? MDA-LDL has already been used, and shown to fail in atheroprotection in uremic, nephrectomized mice (Pedersen *et al.* 2010). Perhaps the identification of a "common epitope" e.g. in bacteria would give new perspective for the vaccination studies aiming for atheroprotective effect.

There seem to be some resemblances between chronic kidney disease and rheumatoid arthritis. Patients suffering from these chronic diseases have more atherosclerotic diseases, and anti-carbamyl-antibodies have been demonstrated in both patient groups. Patients with CKD have persistent low-grade inflammation measured as serum inflammatory biomarkers, such as CRP (Meuwese *et al.* 2011). Patients with rheumatoid arthritis have high risk for amyloidosis, associated with the persistent inflammation, which causes uremia and increased risk of death (Immonen *et al.* 2008, Immonen *et al.* 2011). Do they have some other common factors? Other intriguing questions also arise. Does the carbamyl-epitope play a significant role in other chronic inflammatory diseases? Does MPO-catalyzed carbamylation linked to inflammation induce relevant antibody responses *in vivo*?

7 Conclusions

This study investigated humoral immune response to carbamyl-epitopes in humans and mice under enhanced atherogenesis and carbamylative burden, and the cross-reactivity between carbamylated and oxidation-specific epitopes. The main findings of this thesis study are as follows:

1. Humans, both hemodialysis patients and healthy controls, have plasma antibodies to carbamyl-LDL and carbamylated proteins. IgG levels to carbamylated proteins were higher in dialysis patients compared to controls, and also higher in smokers compared to non-smokers. Human monoclonal Fab antibodies to carbamyl-LDL were cloned from healthy humans by phage display and IgG and IgM antibodies from EBV-infected human B-cells.
2. Cross-reactive humoral antibody response to carbamyl-epitopes and malondialdehyde adducts, and also apoptotic cells containing oxidation-specific epitopes, was observed in humans. Similar cross-reactivity was detected with human plasma antibodies, human monoclonal anti-carbamyl-Fab cloned by phage display, and antibodies secreted by EBV-immortalized human B-cell clones. Hemodialysis patients had more IgG antibodies binding to apoptotic cells than healthy controls. A human Fab inhibited the uptake of modified LDL by macrophages and bound to epitopes found in atherosclerotic lesions.
3. The immunization of $LDLR^{-/-}$ mice with mouse carbamyl-LDL without adjuvant induced a specific IgG immune response not only to carbamyl-LDL but also to carbamylated proteins. Antibody response was observed to carbamyl-epitopes, but also to malondialdehyde-adducts and apoptotic cells. Carbamyl-LDL immunization did not affect the development of atherosclerosis in $LDLR^{-/-}$ mice.

This study revealed new insight into humoral immune response related to increased carbamylative load. Cross-reactive antibodies recognizing carbamyl-epitopes, malondialdehyde-adducts and apoptotic cells may be one step further in revealing the role of carbamylation in atherogenesis.

References

Abbas AK & Lichtman AH (2005) Cellular and Molecular Immunology: Fifth Edition, Updated Edition. Philadelphia, PA, Elsevier Sounders.

Abbas AK, Lichtman AH & Pillai S (2014) Basic immunology: Functions and disorders of the immune system. Philadelphia, Elsevier Sounders.

Afsar B, Turkmen K, Covic A & Kanbay M (2014) An update on coronary artery disease and chronic kidney disease. Int J Nephrol 2014: 767424.

Agrawal S, Gollapudi P, Elahimehr R, Pahl MV & Vaziri ND (2010) Effects of end-stage renal disease and haemodialysis on dendritic cell subsets and basal and LPS-stimulated cytokine production. Nephrol Dial Transplant 25(3): 737–746.

Ainai A, Kawase T, Ida A, Maeda Y, Ohba H, Ikeda Y, Sato H, Takahashi M & Chiba J (2006) Renewal of EBV-hybridoma method: efficient generation of recombinant fully human neutralizing IgG antibodies specific for tetanus toxin by use of tetroma cells. Hum Antibodies 15(4): 139–154.

Ait-Oufella H, Herbin O, Bouaziz JD, Binder CJ, Uyttenhove C, Laurans L, Taleb S, Van Vre E, Esposito B, Vilar J, Sirvent J, Van Snick J, Tedgui A, Tedder TF & Mallat Z (2010) B cell depletion reduces the development of atherosclerosis in mice. J Exp Med 207(8): 1579–1587.

Alahouhala I, Grönhagen-Riska C, Honkanen E, Högström S, Ikäheimo R, Karhapää P, Metsärinne K, Mäkelä S, Räsänen A, Rönnholm K, Salmela K, Säkkinen S, Finne P & Jukkara R (2012) Finnish registry for kidney diseases - Report 2012. URI: www.musili.fi/files/1290/Report2012.pdf.

Ameli S, Hultgårdh-Nilsson A, Regnström J, Calara F, Yano J, Cercek B, Shah PK & Nilsson J (1996) Effect of immunization with homologous LDL and oxidized LDL on early atherosclerosis in hypercholesterolemic rabbits. Arterioscler Thromb Vasc Biol 16(8): 1074–1079.

Andris-Widhopf J, Steinberger P, Fuller R, Rader C & Barbas CF,3rd (2001) Generation of antibody libraries: PCR amplification and assembly of light- and heavy-chain coding sequences. In: Barbas CF,3rd, Burton DR, Scott JK & Silverman GJ (eds) Phage display: A laboratory manual. New York, Cold Spring Harbor Laboratory Press: 9.1–9.113.

Anitschkow N & Chalatow S (1983) Classics in arteriosclerosis research: On experimental cholesterin steatosis and its significance in the origin of some pathological processes by N. Anitschkow and S. Chalatow, translated by Mary Z. Pelias, 1913 Reprinted. Arteriosclerosis 3(2): 178–182.

Ankersmit HJ, Deicher R, Moser B, Teufel I, Roth G, Gerlitz S, Itescu S, Wolner E, Boltz-Nitulescu G & Kovarik J (2001) Impaired T cell proliferation, increased soluble death-inducing receptors and activation-induced T cell death in patients undergoing haemodialysis. Clin Exp Immunol 125(1): 142–148.

Apostolov EO, Basnakian AG, Ok E & Shah SV (2012) Carbamylated low-density lipoprotein: nontraditional risk factor for cardiovascular events in patients with chronic kidney disease. J Ren Nutr 22(1): 134–138.

Apostolov EO, Basnakian AG, Yin X, Ok E & Shah SV (2007) Modified LDLs induce proliferation-mediated death of human vascular endothelial cells through MAPK pathway. Am J Physiol Heart Circ Physiol 292(4): H1836–46.

Apostolov EO, Ok E, Burns S, Nawaz S, Savenka A, Shah S & Basnakian AG (2013) Carbamylated-oxidized LDL: proatherosclerotic effects on endothelial cells and macrophages. J Atheroscler Thromb 20(12): 878–892.

Apostolov EO, Ray D, Alobuia WM, Mikhailova MV, Wang X, Basnakian AG & Shah SV (2011) Endonuclease G mediates endothelial cell death induced by carbamylated LDL. Am J Physiol Heart Circ Physiol 300(6): H1997–2004.

Apostolov EO, Ray D, Savenka AV, Shah SV & Basnakian AG (2010) Chronic uremia stimulates LDL carbamylation and atherosclerosis. J Am Soc Nephrol 21(11): 1852–1857.

Apostolov EO, Shah SV, Ok E & Basnakian AG (2005) Quantification of carbamylated LDL in human sera by a new sandwich ELISA. Clin Chem 51(4): 719–728.

Apostolov EO, Shah SV, Ok E & Basnakian AG (2007) Carbamylated low-density lipoprotein induces monocyte adhesion to endothelial cells through intercellular adhesion molecule-1 and vascular cell adhesion molecule-1. Arterioscler Thromb Vasc Biol 27(4): 826–832.

Apostolov EO, Shah SV, Ray D & Basnakian AG (2009) Scavenger receptors of endothelial cells mediate the uptake and cellular proatherogenic effects of carbamylated LDL. Arterioscler Thromb Vasc Biol 29(10): 1622–1630.

Asci G, Basci A, Shah SV, Basnakian A, Toz H, Ozkahya M, Duman S & Ok E (2008) Carbamylated low-density lipoprotein induces proliferation and increases adhesion molecule expression of human coronary artery smooth muscle cells. Nephrology (Carlton) 13(6): 480–486.

Atamaniuk J, Ruzicka K, Stuhlmeier KM, Karimi A, Eigner M & Mueller MM (2006) Cell-free plasma DNA: a marker for apoptosis during hemodialysis. Clin Chem 52(3): 523–526.

Balion CM, Draisey TF & Thibert RJ (1998) Carbamylated hemoglobin and carbamylated plasma protein in hemodialyzed patients. Kidney Int 53(2): 488–495.

Barbas CF,3rd, Burton DR, Scott JK & Silverman GJ (eds) (2001) Phage Display: A Labotatory Manual. Cold Spring Harbor, NewYork, Cold Spring Harbor Laboratory Press.

Barbas CF,3rd, Kang AS, Lerner RA & Benkovic SJ (1991) Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. Proc Natl Acad Sci U S A 88(18): 7978–7982.

Basnakian AG, Shah SV, Ok E, Altunel E & Apostolov EO (2010) Carbamylated LDL. Adv Clin Chem 51: 25–52.

Bellazzi R, Maggi E, Bellomo G, Finardi G, Gazo A, Nai M, Romanini D, Falaschi F, Frattoni A & Perani G (1993) Low-density lipoprotein oxidation and antioxidized LDL antibodies in peritoneal dialysis patients. Adv Perit Dial 9: 312–317.

Benedetto FA, Tripepi G, Mallamaci F & Zoccali C (2008) Rate of atherosclerotic plaque formation predicts cardiovascular events in ESRD. J Am Soc Nephrol 19(4): 757–763.

Berg AH, Drechsler C, Wenger J, Buccafusca R, Hod T, Kalim S, Ramma W, Parikh SM, Steen H, Friedman DJ, Danziger J, Wanner C, Thadhani R & Karumanchi SA (2013) Carbamylation of serum albumin as a risk factor for mortality in patients with kidney failure. Sci Transl Med 5(175): 175ra29.

Berlyne GM (1998) Carbamylated proteins and peptides in health and in uremia. Nephron 79(2): 125–130.

Bevc S, Hojs R, Ekart R & Hojs-Fabjan T (2006) Atherosclerosis in hemodialysis patients: traditional and nontraditional risk factors. Acta Dermatovenerol Alp Panonica Adriat 15(4): 151–157.

Bhaskaran M, Ranjan R, Shah H, Siu J, Colvin R, Radhakrishnan N, Reddy K, Franki N, Wagner JD & Singhal PC (2002) Lymphopenia in dialysis patients: a preliminary study indicating a possible role of apoptosis. Clin Nephrol 57(3): 221–229.

Bigda JJ & Koszalka P (2013) Waclaw Szybalski's contribution to immunotherapy: HGPRT mutation & HAT selection as first steps to gene therapy and hybrid techniques in mammalian cells. Gene 525(2): 158–161.

Binder CJ (2010) Natural IgM antibodies against oxidation-specific epitopes. J Clin Immunol 30 Suppl 1: S56–60.

Binder CJ, Hörkkö S, Dewan A, Chang MK, Kieu EP, Goodyear CS, Shaw PX, Palinski W, Witztum JL & Silverman GJ (2003) Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between *Streptococcus pneumoniae* and oxidized LDL. Nat Med 9(6): 736–743.

Boaz M, Matas Z, Biro A, Katzir Z, Green M, Fainaru M & Smetana S (1999) Serum malondialdehyde and prevalent cardiovascular disease in hemodialysis. Kidney Int 56(3): 1078–1083.

Bordoni V, Piroddi M, Galli F, de Cal M, Bonello M, Dimitri P, Salvatori G, Ranishta R, Levin N, Tetta C & Ronco C (2006) Oxidant and carbonyl stress-related apoptosis in end-stage kidney disease: impact of membrane flux. Blood Purif 24(1): 149–156.

Bro S, Bentzon JF, Falk E, Andersen CB, Olgaard K & Nielsen LB (2003) Chronic renal failure accelerates atherogenesis in apolipoprotein E-deficient mice. J Am Soc Nephrol 14(10): 2466–2474.

Bro S, Moeller F, Andersen CB, Olgaard K & Nielsen LB (2004) Increased expression of adhesion molecules in uremic atherosclerosis in apolipoprotein-E-deficient mice. J Am Soc Nephrol 15(6): 1495–1503.

Brown MS & Goldstein JL (1983) Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. Annu Rev Biochem 52: 223–261.

Bruggemann M, Winter G, Waldmann H & Neuberger MS (1989) The immunogenicity of chimeric antibodies. J Exp Med 170(6): 2153–2157.

Buzello M, Tornig J, Faulhaber J, Ehmke H, Ritz E & Amann K (2003) The apolipoprotein e knockout mouse: a model documenting accelerated atherogenesis in uremia. J Am Soc Nephrol 14(2): 311–316.

Carracedo J, Merino A, Briceno C, Soriano S, Buendia P, Calleros L, Rodriguez M, Martin-Malo A, Aljama P & Ramirez R (2011) Carbamylated low-density lipoprotein induces oxidative stress and accelerated senescence in human endothelial progenitor cells. FASEB J 25(4): 1314–1322.

Carrero JJ, Hua X, Stenvinkel P, Qureshi AR, Heimburger O, Barany P, Lindholm B & Frostegård J (2009) Low levels of IgM antibodies against phosphorylcholine-A increase mortality risk in patients undergoing haemodialysis. Nephrol Dial Transplant 24(11): 3454–3460.

Chang MK, Bergmark C, Laurila A, Hörkkö S, Han KH, Friedman P, Dennis EA & Witztum JL (1999) Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. Proc Natl Acad Sci U S A 96(11): 6353–6358.

Chang MK, Binder CJ, Miller YI, Subbanagounder G, Silverman GJ, Berliner JA & Witztum JL (2004) Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. J Exp Med 200(11): 1359–1370.

Chaves SS, Daniels D, Cooper BW, Malo-Schlegel S, Macarthur S, Robbins KC, Kobetitsch JF, McDaniel A, D'Avella JF & Alter MJ (2011) Immunogenicity of hepatitis B vaccine among hemodialysis patients: effect of revaccination of non-responders and duration of protection. Vaccine 29(52): 9618–9623.

Ciurana CL & Hack CE (2006) Competitive binding of pentraxins and IgM to newly exposed epitopes on late apoptotic cells. Cell Immunol 239(1): 14–21.

Clackson T, Hoogenboom HR, Griffiths AD & Winter G (1991) Making antibody fragments using phage display libraries. Nature 352(6336): 624–628.

Cohen G & Horl WH (2012) Immune dysfunction in uremia—an update. Toxins (Basel) 4(11): 962–990.

Covic A, Kanbay M, Voroneanu L, Turgut F, Serban DN, Serban IL & Goldsmith DJ (2010) Vascular calcification in chronic kidney disease. Clin Sci (Lond) 119(3): 111–121.

Cybulsky MI & Gimbrone MA,Jr (1991) Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. Science 251(4995): 788–791.

Diepenhorst GM, Ciurana CL, Diaz Padilla N, Boekholdt SM, Krijnen PA, Lagrand WK, Niessen HW & Hack CE (2012) IgM antibodies against apoptotic cells and phosphorylcholine in patients with acute myocardial infarction in relation to infarct size and inflammatory response. Adv Clin Exp Med 21(4): 455–467.

Dorner T, Foster SJ, Farner NL & Lipsky PE (1998) Somatic hypermutation of human immunoglobulin heavy chain genes: targeting of RGYW motifs on both DNA strands. Eur J Immunol 28(10): 3384–3396.

Drüeke TB & Massy ZA (2010) Atherosclerosis in CKD: differences from the general population. Nat Rev Nephrol 6(12): 723–735.

Dudley DD, Chaudhuri J, Bassing CH & Alt FW (2005) Mechanism and control of V(D)J recombination versus class switch recombination: similarities and differences. Adv Immunol 86: 43–112.

Duranti E & Duranti D (2011) Polymethylmethacrylate strengthens antibody response in hemodialysis patients not responding to hepatitis B vaccine: preliminary data. Minerva Med 102(6): 469–474.

El-Gamal D, Holzer M, Gauster M, Schicho R, Binder V, Konya V, Wadsack C, Schuligoi R, Heinemann A & Marsche G (2012) Cyanate is a novel inducer of endothelial ICAM-1 expression. Antioxid Redox Signal 16(2): 129–137.

Esterbauer H, Schaur RJ & Zollner H (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 11(1): 81–128.

Falk E (2006) Pathogenesis of atherosclerosis. J Am Coll Cardiol 47(8 Suppl): C7–12.

Foley RN & Collins AJ (2007) End-stage renal disease in the United States: an update from the United States Renal Data System. J Am Soc Nephrol 18(10): 2644–2648.

Foley RN, Parfrey PS & Sarnak MJ (1998) Clinical epidemiology of cardiovascular disease in chronic renal disease. Am J Kidney Dis 32(5 Suppl 3): S112–9.

Frasca D & Blomberg BB (2011) Aging affects human B cell responses. J Clin Immunol 31(3): 430–435.

Frasca D, Diaz A, Romero M, Landin AM & Blomberg BB (2011) Age effects on B cells and humoral immunity in humans. Ageing Res Rev 10(3): 330–335.

Freigang S, Hörkkö S, Miller E, Witztum JL & Palinski W (1998) Immunization of LDL receptor-deficient mice with homologous malondialdehyde-modified and native LDL reduces progression of atherosclerosis by mechanisms other than induction of high titers of antibodies to oxidative neoepitopes. Arterioscler Thromb Vasc Biol 18(12): 1972–1982.

Frostegård AG, Hua X, Su J, Carrero JJ, Heimburger O, Barany P, Stenvinkel P & Frostegård J (2013) Immunoglobulin (Ig)M antibodies against oxidized cardiolipin but not native cardiolipin are novel biomarkers in haemodialysis patients, associated negatively with mortality. Clin Exp Immunol 174(3): 441–448.

Frostegård J, Svenungsson E, Wu R, Gunnarsson I, Lundberg IE, Klareskog L, Hörkkö S & Witztum JL (2005) Lipid peroxidation is enhanced in patients with systemic lupus erythematosus and is associated with arterial and renal disease manifestations. Arthritis Rheum 52(1): 192–200.

Fruchart JC, Nierman MC, Stroes ES, Kastelein JJ & Duriez P (2004) New risk factors for atherosclerosis and patient risk assessment. Circulation 109(23 Suppl 1): III15–9.

Gagnon RF & Duguid WP (1983) A reproducible model for chronic renal failure in the mouse. Urol Res 11(1): 11–14.

Galle J, Heinloth A, Wanner C & Heermeier K (2001) Dual effect of oxidized LDL on cell cycle in human endothelial cells through oxidative stress. Kidney Int Suppl 78: S120–3.

Galli F, Ghibelli L, Buoncristiani U, Bordoni V, D'Intini V, Benedetti S, Canestrari F, Ronco C & Floridi A (2003) Mononuclear leukocyte apoptosis in haemodialysis patients: the role of cell thiols and vitamin E. Nephrol Dial Transplant 18(8): 1592–1600.

Glass CK & Witztum JL (2001) Atherosclerosis. the road ahead. Cell 104(4): 503–516.

Gonen B, Cole T & Hahm KS (1983) The interaction of carbamylated low-density lipoprotein with cultured cells. Studies with human fibroblasts, rat peritoneal macrophages and human monocyte-derived macrophages. Biochim Biophys Acta 754(2): 201–207.

Gonen B, Goldberg AP, Harter HR & Schonfeld G (1985) Abnormal cell-interactive properties of low-density lipoproteins isolated from patients with chronic renal failure. Metabolism 34(1): 10–14.

Green LL, Hardy MC, Maynard-Currie CE, Tsuda H, Louie DM, Mendez MJ, Abderrahim H, Noguchi M, Smith DH, Zeng Y, David NE, Sasai H, Garza D, Brenner DG, Hales JF, McGuinness RP, Capon DJ, Klapholz S & Jakobovits A (1994) Antigen-specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs. Nat Genet 7(1): 13–21.

Habeeb AF (1966) Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. Anal Biochem 14(3): 328–336.

Hansson GK (2005) Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 352(16): 1685–1695.

Havel RJ, Eder HA & Bragdon JH (1955) The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest 34(9): 1345–1353.

Heidenreich S, Schmidt M, Bachmann J & Harrach B (1996) Apoptosis of monocytes cultured from long-term hemodialysis patients. Kidney Int 49(3): 792–799.

Heinloth A, Heermeier K, Raff U, Wanner C & Galle J (2000) Stimulation of NADPH oxidase by oxidized low-density lipoprotein induces proliferation of human vascular endothelial cells. J Am Soc Nephrol 11(10): 1819–1825.

Henriksen T, Mahoney EM & Steinberg D (1981) Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by receptors for acetylated low density lipoproteins. Proc Natl Acad Sci U S A 78(10): 6499–6503.

Hessler JR, Morel DW, Lewis LJ & Chisolm GM (1983) Lipoprotein oxidation and lipoprotein-induced cytotoxicity. Arteriosclerosis 3(3): 215–222.

Hevonoja T, Pentikäinen MO, Hyvönen MT, Kovanen PT & Ala-Korpela M (2000) Structure of low density lipoprotein (LDL) particles: basis for understanding molecular changes in modified LDL. Biochim Biophys Acta 1488(3): 189–210.

Hill GE, Miller JA, Baxter BT, Klassen LW, Duryee MJ, Tuma DJ & Thiele GM (1998) Association of malondialdehyde-acetaldehyde (MAA) adducted proteins with atherosclerotic-induced vascular inflammatory injury. Atherosclerosis 141(1): 107–116.

Holzer M, Gauster M, Pfeifer T, Wadsack C, Fauler G, Stiegler P, Koefeler H, Beubler E, Schuligoi R, Heinemann A & Marsche G (2011) Protein carbamylation renders high-density lipoprotein dysfunctional. Antioxid Redox Signal 14(12): 2337–2346.

Honjo T (1983) Immunoglobulin genes. Annu Rev Immunol 1: 499–528.

Hoogenboom HR, Griffiths AD, Johnson KS, Chiswell DJ, Hudson P & Winter G (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. Nucleic Acids Res 19(15): 4133–4137.

Hörkkö S, Binder CJ, Shaw PX, Chang MK, Silverman G, Palinski W & Witztum JL (2000) Immunological responses to oxidized LDL. Free Radic Biol Med 28(12): 1771–1779.

Hörkkö S, Bird DA, Miller E, Itabe H, Leitinger N, Subbanagounder G, Berliner JA, Friedman P, Dennis EA, Curtiss LK, Palinski W & Witztum JL (1999) Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. J Clin Invest 103(1): 117–128.

Hörkkö S, Huttunen K, Kervinen K & Kesäniemi YA (1994) Decreased clearance of uraemic and mildly carbamylated low-density lipoprotein. Eur J Clin Invest 24(2): 105–113.

Hörkkö S, Huttunen K & Kesäniemi YA (1995) Decreased clearance of low-density lipoprotein in uremic patients under dialysis treatment. Kidney Int 47(6): 1732–1740.

Hörkkö S, Huttunen K, Korhonen T & Kesäniemi YA (1994) Decreased clearance of low-density lipoprotein in patients with chronic renal failure. Kidney Int 45(2): 561–570.

Hörkkö S, Savolainen MJ, Kervinen K & Kesäniemi YA (1992) Carbamylation-induced alterations in low-density lipoprotein metabolism. Kidney Int 41(5): 1175–1181.

Hulthe J (2004) Antibodies to oxidized LDL in atherosclerosis development--clinical and animal studies. Clin Chim Acta 348(1–2): 1–8.

Husgafvel-Pursiainen K, Sorsa M, Engström K & Einistö P (1987) Passive smoking at work: biochemical and biological measures of exposure to environmental tobacco smoke. Int Arch Occup Environ Health 59(4): 337–345.

Immonen K, Finne P, Gronhagen-Riska C, Pettersson T, Klaukka T, Kautiainen H & Hakala M (2011) A marked decline in the incidence of renal replacement therapy for amyloidosis associated with inflammatory rheumatic diseases - data from nationwide registries in Finland. Amyloid 18(1): 25–28.

Immonen K, Finne P, Hakala M, Kautiainen H, Pettersson T & Grönhagen-Riska C (2008) No improvement in survival of patients with amyloidosis associated with inflammatory rheumatic diseases -- data from the Finnish national registry for kidney diseases. J Rheumatol 35(7): 1334–1338.

Jaber BL, Cendoroglo M, Balakrishnan VS, Perianayagam MC, King AJ & Pereira BJ (2001) Apoptosis of leukocytes: basic concepts and implications in uremia. Kidney Int Suppl 78: S197–205.

Jaisson S, Gorisse L, Pietrement C & Gillery P (2012) Quantification of plasma homocitrulline using hydrophilic interaction liquid chromatography (HILIC) coupled to tandem mass spectrometry. Anal Bioanal Chem 402(4): 1635–1641.

Jeon YE, Seo CW, Yu ES, Lee CJ, Park SG & Jang YJ (2007) Characterization of human monoclonal autoantibody Fab fragments against oxidized LDL. Mol Immunol 44(5): 827–836.

Jones PT, Dear PH, Foote J, Neuberger MS & Winter G (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. Nature 321(6069): 522–525.

Karpas A, Dremucheva A & Czepulkowski BH (2001) A human myeloma cell line suitable for the generation of human monoclonal antibodies. Proc Natl Acad Sci U S A 98(4): 1799–1804.

Karpas A, Harder L, Czepulkowski B, Bloxham D, Saward R, Dremucheva A & Siebert R (2005) Studies of four new human myeloma cell lines. Leuk Lymphoma 46(1): 101–112.

Karvonen J, Päivänsalo M, Kesäniemi YA & Hörkkö S (2003) Immunoglobulin M type of autoantibodies to oxidized low-density lipoprotein has an inverse relation to carotid artery atherosclerosis. Circulation 108(17): 2107–2112.

Kaveri SV, Silverman GJ & Bayry J (2012) Natural IgM in immune equilibrium and harnessing their therapeutic potential. J Immunol 188(3): 939–945.

Kaysen GA & Don BR (2003) Factors that affect albumin concentration in dialysis patients and their relationship to vascular disease. Kidney Int Suppl (84)(84): S94–7.

Kellokoski E, Kummu O, Serpi R, Lehenkari P, Ukkola O, Kesäniemi YA & Hörkkö S (2009) Ghrelin vaccination decreases plasma MCP-1 level in LDLR(-/-)-mice. Peptides 30(12): 2292–2300.

Khallou-Laschet J, Tupin E, Caligiuri G, Poirier B, Thieblemont N, Gaston AT, Vandaele M, Bleton J, Tchapla A, Kaveri SV, Rudling M & Nicoletti A (2006) Atheroprotective effect of adjuvants in apolipoprotein E knockout mice. Atherosclerosis 184(2): 330–341.

Kim SJ, Gershov D, Ma X, Brot N & Elkon KB (2002) I-PLA(2) activation during apoptosis promotes the exposure of membrane lysophosphatidylcholine leading to binding by natural immunoglobulin M antibodies and complement activation. J Exp Med 196(5): 655–665.

Kodama T, Reddy P, Kishimoto C & Krieger M (1988) Purification and characterization of a bovine acetyl low density lipoprotein receptor. Proc Natl Acad Sci U S A 85(23): 9238–9242.

Koeth RA, Kalantar-Zadeh K, Wang Z, Fu X, Tang WH & Hazen SL (2013) Protein Carbamylation Predicts Mortality in ESRD. J Am Soc Nephrol 24(5): 853–861.

Köhler G & Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256(5517): 495–497.

Kraus LM & Kraus AP, Jr (2001) Carbamoylation of amino acids and proteins in uremia. Kidney Int Suppl 78: S102–7.

Kyaw T, Tay C, Krishnamurthi S, Kanellakis P, Agrotis A, Tipping P, Bobik A & Toh BH (2011) B1a B lymphocytes are atheroprotective by secreting natural IgM that increases IgM deposits and reduces necrotic cores in atherosclerotic lesions. Circ Res 109(8): 830–840.

Levey AS, Atkins R, Coresh J, Cohen EP, Collins AJ, Eckardt KU, Nahas ME, Jaber BL, Jadoul M, Levin A, Powe NR, Rossert J, Wheeler DC, Lameire N & Eknoyan G (2007) Chronic kidney disease as a global public health problem: approaches and initiatives - a position statement from Kidney Disease Improving Global Outcomes. Kidney Int 72(3): 247–259.

Levey AS & Coresh J (2003) Should the K/DOQI definition of chronic kidney disease be changed? Am J Kidney Dis 42(4): 626–630.

Levey AS, Coresh J, Balk E, Kausz AT, Levin A, Steffes MW, Hogg RJ, Perrone RD, Lau J, Eknoyan G & National Kidney Foundation (2003) National Kidney Foundation practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Ann Intern Med 139(2): 137–147.

Levy R & Dilley J (1978) Rescue of immunoglobulin secretion from human neoplastic lymphoid cells by somatic cell hybridization. Proc Natl Acad Sci U S A 75(5): 2411–2415.

Levy R, Dilley J, Sikora K & Kucherlapati R (1978) Mouse-human hybridomas. The conversion of non-secreting human B cells into Ig secretors. Curr Top Microbiol Immunol 81: 170–172.

Lewis MJ, Malik TH, Ehrenstein MR, Boyle JJ, Botto M & Haskard DO (2009) Immunoglobulin M is required for protection against atherosclerosis in low-density lipoprotein receptor-deficient mice. Circulation 120(5): 417–426.

Libby P (2002) Inflammation in atherosclerosis. Nature 420(6917): 868–874.

Libby P (2012) Inflammation in atherosclerosis. Arterioscler Thromb Vasc Biol 32(9): 2045–2051.

Libby P, Ridker PM & Hansson GK (2011) Progress and challenges in translating the biology of atherosclerosis. Nature 473(7347): 317–325.

Lindner A, Charra B, Sherrard DJ & Scribner BH (1974) Accelerated atherosclerosis in prolonged maintenance hemodialysis. N Engl J Med 290(13): 697–701.

Locatelli F, Pozzoni P, Tentori F & del Vecchio L (2003) Epidemiology of cardiovascular risk in patients with chronic kidney disease. Nephrol Dial Transplant 18 Suppl 7: vii2–9.

Lonberg N (2005) Human antibodies from transgenic animals. Nat Biotechnol 23(9): 1117–1125.

Lonberg N, Taylor LD, Harding FA, Trounstine M, Higgins KM, Schramm SR, Kuo CC, Mashayekh R, Wymore K & McCabe JG (1994) Antigen-specific human antibodies from mice comprising four distinct genetic modifications. Nature 368(6474): 856–859.

Lopes-Virella MF & Virella G (2010) Clinical significance of the humoral immune response to modified LDL. Clin Immunol 134(1): 55–65.

Lopes-Virella MF, Virella G, Orchard TJ, Koskinen S, Evans RW, Becker DJ & Forrest KY (1999) Antibodies to oxidized LDL and LDL-containing immune complexes as risk factors for coronary artery disease in diabetes mellitus. Clin Immunol 90(2): 165–172.

Maggi E, Bellazzi R, Falaschi F, Frattoni A, Perani G, Finardi G, Gazo A, Nai M, Romanini D & Bellomo G (1994a) Enhanced LDL oxidation in uremic patients: an additional mechanism for accelerated atherosclerosis? Kidney Int 45(3): 876–883.

Maggi E, Bellazzi R, Gazo A, Seccia M & Bellomo G (1994b) Autoantibodies against oxidatively-modified LDL in uremic patients undergoing dialysis. Kidney Int 46(3): 869–876.

Maggi E, Chiesa R, Melissano G, Castellano R, Astore D, Grossi A, Finardi G & Bellomo G (1994c) LDL oxidation in patients with severe carotid atherosclerosis. A study of in vitro and in vivo oxidation markers. Arterioscler Thromb 14(12): 1892–1899.

Manson JJ, Mauri C & Ehrenstein MR (2005) Natural serum IgM maintains immunological homeostasis and prevents autoimmunity. Springer Semin Immunopathol 26(4): 425–432.

Marks JD, Hoogenboom HR, Bonnert TP, McCafferty J, Griffiths AD & Winter G (1991) By-passing immunization. Human antibodies from V-gene libraries displayed on phage. J Mol Biol 222(3): 581–597.

Martin-Malo A, Carracedo J, Ramirez R, Rodriguez-Benot A, Soriano S, Rodriguez M & Aljama P (2000) Effect of uremia and dialysis modality on mononuclear cell apoptosis. J Am Soc Nephrol 11(5): 936–942.

Matsumoto Y, Shinzato T, Amano I, Takai I, Kimura Y, Morita H, Miwa M, Nakane K, Yoshikai Y & Maeda K (1995) Relationship between susceptibility to apoptosis and Fas expression in peripheral blood T cells from uremic patients: a possible mechanism for lymphopenia in chronic renal failure. Biochem Biophys Res Commun 215(1): 98–105.

Matthias P & Rolink AG (2005) Transcriptional networks in developing and mature B cells. Nat Rev Immunol 5(6): 497–508.

McCafferty J, Griffiths AD, Winter G & Chiswell DJ (1990) Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348(6301): 552–554.

McClellan W, Aronoff SL, Bolton WK, Hood S, Lorber DL, Tang KL, Tse TF, Wasserman B & Leiserowitz M (2004) The prevalence of anemia in patients with chronic kidney disease. Curr Med Res Opin 20(9): 1501–1510.

Meuwese CL, Stenvinkel P, Dekker FW & Carrero JJ (2011) Monitoring of inflammation in patients on dialysis: forewarned is forearmed. Nat Rev Nephrol 7(3): 166–176.

Morrison SL, Johnson MJ, Herzenberg LA & Oi VT (1984) Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. Proc Natl Acad Sci U S A 81(21): 6851–6855.

Muller PC, Anink J, Shi J, Levarht EW, Reinards TH, Otten MH, van Tol MJ, Jol-van der Zijde CM, Brinkman DM, Allaart CF, Hoppenreijs EP, Koopman-Keemink Y, Kamphuis SS, Dolman K, van den Berg JM, van Rossum MA, van Suijlekom-Smit LW, Schilham MW, Huizinga TW, Toes RE, Ten Cate R & Trouw LA (2013) Anticarbamylated protein (anti-CarP) antibodies are present in sera of juvenile idiopathic arthritis (JIA) patients. Ann Rheum Dis 72(12): 2053–2055.

Napoli C, D'Armiento FP, Mancini FP, Postiglione A, Witztum JL, Palumbo G & Palinski W (1997) Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal hypercholesterolemia. Intimal accumulation of low density lipoprotein and its oxidation precede monocyte recruitment into early atherosclerotic lesions. J Clin Invest 100(11): 2680–2690.

Nelson AL, Dhimolea E & Reichert JM (2010) Development trends for human monoclonal antibody therapeutics. Nat Rev Drug Discov 9(10): 767–774.

Neu AM (2012) Immunizations in children with chronic kidney disease. Pediatr Nephrol 27(8): 1257–1263.

Newton AH & Benedict SH (2014) Low density lipoprotein promotes human naive T cell differentiation to Th1 cells. Hum Immunol 75(7): 621–628.

Nilsson J, Calara F, Regnström J, Hultgårdh-Nilsson A, Ameli S, Cercek B & Shah PK (1997) Immunization with homologous oxidized low density lipoprotein reduces neointimal formation after balloon injury in hypercholesterolemic rabbits. J Am Coll Cardiol 30(7): 1886–1891.

Nissinen AE, Laitinen LM, Kakko S, Helander A, Savolainen MJ & Hörkkö S (2012) Low plasma antibodies specific for phosphatidylethanol in alcohol abusers and patients with alcoholic pancreatitis. Addict Biol 17(6): 1057–1067.

Ok E, Basnakian AG, Apostolov EO, Barri YM & Shah SV (2005) Carbamylated low-density lipoprotein induces death of endothelial cells: a link to atherosclerosis in patients with kidney disease. Kidney Int 68(1): 173–178.

Olsson L & Kaplan HS (1980) Human-human hybridomas producing monoclonal antibodies of predefined antigenic specificity. Proc Natl Acad Sci U S A 77(9): 5429–5431.

Osbourn J, Jermutus L & Duncan A (2003) Current methods for the generation of human antibodies for the treatment of autoimmune diseases. Drug Discov Today 8(18): 845–851.

Pahl MV, Gollapudi S, Sepassi L, Gollapudi P, Elahimehr R & Vaziri ND (2010) Effect of end-stage renal disease on B-lymphocyte subpopulations, IL-7, BAFF and BAFF receptor expression. Nephrol Dial Transplant 25(1): 205–212.

Palinski W, Hörkkö S, Miller E, Steinbrecher UP, Powell HC, Curtiss LK & Witztum JL (1996) Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. J Clin Invest 98(3): 800–814.

Palinski W, Miller E & Witztum JL (1995) Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. Proc Natl Acad Sci U S A 92(3): 821–825.

Palinski W, Ord VA, Plump AS, Breslow JL, Steinberg D & Witztum JL (1994) ApoE-deficient mice are a model of lipoprotein oxidation in atherogenesis. Demonstration of oxidation-specific epitopes in lesions and high titers of autoantibodies to malondialdehyde-lysine in serum. Arterioscler Thromb 14(4): 605–616.

Palinski W, Rosenfeld ME, Ylä-Herttuala S, Gurtner GC, Socher SS, Butler SW, Parthasarathy S, Carew TE, Steinberg D & Witztum JL (1989) Low density lipoprotein undergoes oxidative modification in vivo. Proc Natl Acad Sci U S A 86(4): 1372–1376.

Palinski W, Tangirala RK, Miller E, Young SG & Witztum JL (1995) Increased autoantibody titers against epitopes of oxidized LDL in LDL receptor-deficient mice with increased atherosclerosis. Arterioscler Thromb Vasc Biol 15(10): 1569–1576.

Palinski W, Ylä-Herttuala S, Rosenfeld ME, Butler SW, Socher SA, Parthasarathy S, Curtiss LK & Witztum JL (1990) Antisera and monoclonal antibodies specific for epitopes generated during oxidative modification of low density lipoprotein. Arteriosclerosis 10(3): 325–335.

Payne RA (2012) Cardiovascular risk. Br J Clin Pharmacol 74(3): 396–410.

Pedersen TX, Binder CJ, Fredrikson GN, Nilsson J, Bro S & Nielsen LB (2010) The pro-inflammatory effect of uraemia overrules the anti-atherogenic potential of immunization with oxidized LDL in apoE-/- mice. Nephrol Dial Transplant 25(8): 2486–2491.

Peres LA & Percio PP (2014) Mineral and bone disorder and vascular calcification in patients with chronic kidney disease. J Bras Nefrol 36(2): 201–207.

Piedrahita JA, Zhang SH, Hagaman JR, Oliver PM & Maeda N (1992) Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. Proc Natl Acad Sci U S A 89(10): 4471–4475.

Pietrement C, Gorisse L, Jaisson S & Gillery P (2013) Chronic increase of urea leads to carbamylated proteins accumulation in tissues in a mouse model of CKD. PLoS One 8(12): e82506.

Plump AS, Smith JD, Hayek T, Aalto-Setälä K, Walsh A, Verstuyft JG, Rubin EM & Breslow JL (1992) Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. Cell 71(2): 343–353.

Prinsen BH & de Sain-van der Velden MG (2004) Albumin turnover: experimental approach and its application in health and renal diseases. Clin Chim Acta 347(1–2): 1–14.

Rada C, Ehrenstein MR, Neuberger MS & Milstein C (1998) Hot spot focusing of somatic hypermutation in MSH2-deficient mice suggests two stages of mutational targeting. Immunity 9(1): 135–141.

Randolph GJ (2014) Mechanisms That Regulate Macrophage Burden in Atherosclerosis. Circ Res 114(11): 1757–1771.

Ranjan R, Shah H, Siu J, Varghese E, Bhaskaran M, Reddy K, Kapasi AA, Wagner JD & Singhal PC (2002) Monocyte apoptosis in dialysis patients is Fas ligand-mediated. Clin Nephrol 58(6): 423–430.

Razzaque MS (2013) Phosphate toxicity and vascular mineralization. Contrib Nephrol 180: 74–85.

Reefman E, Horst G, Nijk MT, Limburg PC, Kallenberg CG & Bijl M (2007) Opsonization of late apoptotic cells by systemic lupus erythematosus autoantibodies inhibits their uptake via an Fcgamma receptor-dependent mechanism. Arthritis Rheum 56(10): 3399–3411.

Riechmann L, Clark M, Waldmann H & Winter G (1988) Reshaping human antibodies for therapy. Nature 332(6162): 323–327.

del Rincon ID, Williams K, Stern MP, Freeman GL & Escalante A (2001) High incidence of cardiovascular events in a rheumatoid arthritis cohort not explained by traditional cardiac risk factors. Arthritis Rheum 44(12): 2737–2745.

Roder JC, Cole SP & Kozbor D (1986) The EBV-hybridoma technique. Methods Enzymol 121: 140–167.

Rosen A, Gergely P, Jondal M, Klein G & Britton S (1977) Polyclonal Ig production after Epstein-Barr virus infection of human lymphocytes in vitro. Nature 267(5606): 52–54.

Rosenfeld ME, Palinski W, Ylä-Herttuala S, Butler S & Witztum JL (1990) Distribution of oxidation specific lipid-protein adducts and apolipoprotein B in atherosclerotic lesions of varying severity from WHHL rabbits. Arteriosclerosis 10(3): 336–349.

Ross R (1999) Atherosclerosis--an inflammatory disease. N Engl J Med 340(2): 115–126.

Ross R, Glomset J & Harker L (1977) Response to injury and atherogenesis. Am J Pathol 86(3): 675–684.

Ross R & Glomset JA (1973) Atherosclerosis and the arterial smooth muscle cell: Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. Science 180(4093): 1332–1339.

Roxborough HE & Young IS (1995) Carbamylation of proteins and atherogenesis in renal failure. Med Hypotheses 45(2): 125–128.

Salonen JT, Ylä-Herttuala S, Yamamoto R, Butler S, Korpela H, Salonen R, Nyyssönen K, Palinski W & Witztum JL (1992) Autoantibody against oxidised LDL and progression of carotid atherosclerosis. Lancet 339(8798): 883–887.

Schroeder HW,Jr & Cavacini L (2010) Structure and function of immunoglobulins. J Allergy Clin Immunol 125(2 Suppl 2): S41–52.

Scott JK & Smith GP (1990) Searching for peptide ligands with an epitope library. Science 249(4967): 386–390.

Seidman JG, Leder A, Nau M, Norman B & Leder P (1978) Antibody diversity. Science 202(4363): 11–17.

Seidman JG, Nau MM, Norman B, Kwan SP, Scharff M & Leder P (1980) Immunoglobulin V/J recombination is accompanied by deletion of joining site and variable region segments. Proc Natl Acad Sci U S A 77(10): 6022–6026.

Shaw PX, Hörkkö S, Chang MK, Curtiss LK, Palinski W, Silverman GJ & Witztum JL (2000) Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. J Clin Invest 105(12): 1731–1740.

Shaw PX, Hörkkö S, Tsimikas S, Chang MK, Palinski W, Silverman GJ, Chen PP & Witztum JL (2001) Human-derived anti-oxidized LDL autoantibody blocks uptake of oxidized LDL by macrophages and localizes to atherosclerotic lesions in vivo. Arterioscler Thromb Vasc Biol 21(8): 1333–1339.

Shi J, Knevel R, Suwannalai P, van der Linden MP, Janssen GM, van Veelen PA, Levarht NE, van der Helm-van Mil AH, Cerami A, Huizinga TW, Toes RE & Trouw LA (2011) Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. Proc Natl Acad Sci U S A 108(42): 17372–17377.

Shi J, van de Stadt LA, Levarht EW, Huizinga TW, Toes RE, Trouw LA & van Schaardenburg D (2013) Anti-carbamylated protein antibodies are present in arthralgia patients and predict the development of rheumatoid arthritis. Arthritis Rheum 65(4): 911–915.

Sirpal S (2009) Myeloperoxidase-mediated lipoprotein carbamylation as a mechanistic pathway for atherosclerotic vascular disease. Clin Sci (Lond) 116(9): 681–695.

Smith GP (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228(4705): 1315–1317.

Solomon DH, Goodson NJ, Katz JN, Weinblatt ME, Avorn J, Setoguchi S, Canning C & Schneeweiss S (2006) Patterns of cardiovascular risk in rheumatoid arthritis. Ann Rheum Dis 65(12): 1608–1612.

Son JN, Lho Y, Shin S, Kwon SH, Moon KC & Ha E (2011) Carbamylated low-density lipoprotein increases reactive oxygen species (ROS) and apoptosis via lectin-like oxidized LDL receptor (LOX-1) mediated pathway in human umbilical vein endothelial cells. Int J Cardiol 146(3): 428–430.

Speer T, Owala FO, Holy EW, Zewinger S, Frenzel FL, Stahli BE, Razavi M, Triem S, Cvija H, Rohrer L, Seiler S, Heine GH, Jankowski V, Jankowski J, Camici GG, Akhmedov A, Fliser D, Luscher TF & Tanner FC (In press) Carbamylated low-density lipoprotein induces endothelial dysfunction. Eur Heart J. First published online March 21, 2014.

Stark GR (1964) On the Reversible Reaction of Cyanate with Sulfhydryl Groups and the Determination of Nh2-Terminal Cysteine and Cystine in Proteins. J Biol Chem 239: 1411–1414.

Stark GR (1965) Reactions of cyanate with functional groups of proteins. 3. Reactions with amino and carboxyl groups. Biochemistry 4(6): 1030–1036.

Stark GR, Stein WH & Moore S (1960) Reactions of the cyanate present in aqueous urea with amino acids and proteins. J Biol Chem 235(11): 3177–3181.

Steinbrecher UP, Fisher M, Witztum JL & Curtiss LK (1984a) Immunogenicity of homologous low density lipoprotein after methylation, ethylation, acetylation, or carbamylation: generation of antibodies specific for derivatized lysine. J Lipid Res 25(10): 1109–1116.

Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL & Steinberg D (1984b) Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. Proc Natl Acad Sci U S A 81(12): 3883–3887.

Steinitz M, Klein G, Koskimies S & Makel O (1977) EB virus-induced B lymphocyte cell lines producing specific antibody. Nature 269(5627): 420–422.

Su J, Georgiades A, Wu R, Thulin T, de Faire U & Frostegård J (2006) Antibodies of IgM subclass to phosphorylcholine and oxidized LDL are protective factors for atherosclerosis in patients with hypertension. Atherosclerosis 188(1): 160–166.

Sugimoto M, Furuichi Y, Ide T & Goto M (1999) Incorrect us of "immortalization" for B-lymphoblastoid cell lines transformed by Epstein-Barr virus. J Virol 73(11): 9690–9691.

Szybalska EH & Szybalski W (1962) Genetics of human cess line. IV. DNA-mediated heritable transformation of a biochemical trait. Proc Natl Acad Sci U S A 48: 2026–2034.

Tabas I, Williams KJ & Boren J (2007) Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. Circulation 116(16): 1832–1844.

Tamer L, Sucu N, Polat G, Ercan B, Aytacoglu B, Yucebilgic G, Unlu A, Dikmengil M & Atik U (2002) Decreased serum total antioxidant status and erythrocyte-reduced glutathione levels are associated with increased serum malondialdehyde in atherosclerotic patients. Arch Med Res 33(3): 257–260.

Tangirala RK, Rubin EM & Palinski W (1995) Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. J Lipid Res 36(11): 2320–2328.

Toda T & Sugimoto M (2003) Proteome analysis of Epstein-Barr virus-transformed B-lymphoblasts and the proteome database. J Chromatogr B Analyt Technol Biomed Life Sci 787(1): 197–206.

Tonegawa S (1983) Somatic generation of antibody diversity. Nature 302(5909): 575–581.

Trouw LA, Huizinga TW & Toes RE (2013) Autoimmunity in rheumatoid arthritis: different antigens--common principles. Ann Rheum Dis 72 Suppl 2: ii132–6.

Tsimikas S, Brilakis ES, Lennon RJ, Miller ER, Witztum JL, McConnell JP, Kornman KS & Berger PB (2007) Relationship of IgG and IgM autoantibodies to oxidized low density lipoprotein with coronary artery disease and cardiovascular events. J Lipid Res 48(2): 425–433.

Tsimikas S, Willeit P, Willeit J, Santer P, Mayr M, Xu Q, Mayr A, Witztum JL & Kiechl S (2012) Oxidation-specific biomarkers, prospective 15-year cardiovascular and stroke outcomes, and net reclassification of cardiovascular events. J Am Coll Cardiol 60(21): 2218–2229.

Tuma DJ, Kearley ML, Thiele GM, Worrall S, Haver A, Klassen LW & Sorrell MF (2001) Elucidation of reaction scheme describing malondialdehyde-acetaldehyde-protein adduct formation. Chem Res Toxicol 14(7): 822–832.

Tuma DJ, Thiele GM, Xu D, Klassen LW & Sorrell MF (1996) Acetaldehyde and malondialdehyde react together to generate distinct protein adducts in the liver during long-term ethanol administration. Hepatology 23(4): 872–880.

Tuominen A, Miller YI, Hansen LF, Kesäniemi YA, Witztum JL & Hörkkö S (2006) A natural antibody to oxidized cardiolipin binds to oxidized low-density lipoprotein, apoptotic cells, and atherosclerotic lesions. Arterioscler Thromb Vasc Biol 26(9): 2096–2102.

Turunen S, Koivula MK, Risteli L & Risteli J (2010) Anticitrulline antibodies can be caused by homocitrulline-containing proteins in rabbits. Arthritis Rheum 62(11): 3345–3352.

Turunen SP, Kummu O, Harila K, Veneskoski M, Soliymani R, Baumann M, Pussinen PJ & Hörkkö S (2012) Recognition of *Porphyromonas gingivalis* gingipain epitopes by natural IgM binding to malondialdehyde modified low-density lipoprotein. PLoS One 7(4): e34910.

Turunen SP, Kummu O, Wang C, Harila K, Mattila R, Sahlman M, Pussinen PJ & Hörkkö S (In press) Immunization with malondialdehyde-modified low-density lipoprotein (LDL) reduces atherosclerosis in LDL receptor-deficient mice challenged with Porphyromonas gingivalis. Innate Immun. First published online Aug 17, 2014.

Vaisbourd M, Ignatovich O, Dremucheva A, Karpas A & Winter G (2001) Molecular characterization of human monoclonal antibodies derived from fusions of tonsil lymphocytes with a human myeloma cell line. Hybrid Hybridomics 20(5–6): 287–292.

Vay D, Rigamonti C, Vidali M, Mottaran E, Alchera E, Occhino G, Sartori M & Albano E (2006) Anti-phospholipid antibodies associated with alcoholic liver disease target oxidized phosphatidylserine on apoptotic cell plasma membranes. J Hepatol 44(1): 183–189.

Veneskoski M, Turunen SP, Kummu O, Nissinen A, Rannikko S, Levonen AL & Hörkkö S (2011) Specific recognition of malondialdehyde and malondialdehyde acetaldehyde adducts on oxidized LDL and apoptotic cells by complement anaphylatoxin C3a. Free Radic Biol Med 51(4): 834–843.

Ventura HO (2000) Profiles in cardiology. Rudolph Virchow and cellular pathology. Clin Cardiol 23(7): 550–552.

Viigimaa M, Abina J, Zemtsovskaya G, Tikhaze A, Konovalova G, Kumskova E & Lankin V (2010) Malondialdehyde-modified low-density lipoproteins as biomarker for atherosclerosis. Blood Press 19(3): 164–168.

Vos FE, Schollum JB, Coulter CV, Doyle TC, Duffull SB & Walker RJ (2011) Red blood cell survival in long-term dialysis patients. Am J Kidney Dis 58(4): 591–598.

Wang C, Turunen SP, Kummu O, Veneskoski M, Lehtimäki J, Nissinen AE & Hörkkö S (2013) Natural antibodies of newborns recognize oxidative stress -related malondialdehyde acetaldehyde adducts on apoptotic cells and atherosclerotic plaques. Int Immunol 25(10): 575–587.

Wang Z, Nicholls SJ, Rodriguez ER, Kummu O, Hörkkö S, Barnard J, Reynolds WF, Topol EJ, DiDonato JA & Hazen SL (2007) Protein carbamylation links inflammation, smoking, uremia and atherogenesis. Nat Med 13(10): 1176–1184.

Weber C & Noels H (2011) Atherosclerosis: current pathogenesis and therapeutic options. Nat Med 17(11): 1410–1422.

Weisgraber KH, Innerarity TL & Mahley RW (1978) Role of lysine residues of plasma lipoproteins in high affinity binding to cell surface receptors on human fibroblasts. J Biol Chem 253(24): 9053–9062.

Wever R, Kast WM, Kasinoedin JH & Boelens R (1982) The peroxidation of thiocyanate catalysed by myeloperoxidase and lactoperoxidase. Biochim Biophys Acta 709(2): 212–219.

Williams KJ & Tabas I (1995) The response-to-retention hypothesis of early atherogenesis. Arterioscler Thromb Vasc Biol 15(5): 551–561.

Williams KJ & Tabas I (1998) The response-to-retention hypothesis of atherogenesis reinforced. Curr Opin Lipidol 9(5): 471–474.

Witztum JL, Steinbrecher UP, Fisher M & Kesäniemi A (1983) Nonenzymatic glucosylation of homologous low density lipoprotein and albumin renders them immunogenic in the guinea pig. Proc Natl Acad Sci U S A 80(9): 2757–2761.

Witztum JL, Steinbrecher UP, Kesäniemi YA & Fisher M (1984) Autoantibodies to glucosylated proteins in the plasma of patients with diabetes mellitus. Proc Natl Acad Sci U S A 81(10): 3204–3208.

Xu Q, Dietrich H, Steiner HJ, Gown AM, Schoel B, Mikuz G, Kaufmann SH & Wick G (1992) Induction of arteriosclerosis in normocholesterolemic rabbits by immunization with heat shock protein 65. Arterioscler Thromb 12(7): 789–799.

Yamashita M, Katakura Y & Shirahata S (2007) Recent advances in the generation of human monoclonal antibody. Cytotechnology 55(2–3): 55–60.

Ylä-Herttuala S, Palinski W, Butler SW, Picard S, Steinberg D & Witztum JL (1994) Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL. Arterioscler Thromb 14(1): 32–40.

Ylä-Herttuala S, Palinski W, Rosenfeld ME, Parthasarathy S, Carew TE, Butler S, Witztum JL & Steinberg D (1989) Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. J Clin Invest 84(4): 1086–1095.

Zhou X, Caligiuri G, Hamsten A, Lefvert AK & Hansson GK (2001) LDL immunization induces T-cell-dependent antibody formation and protection against atherosclerosis. Arterioscler Thromb Vasc Biol 21(1): 108–114.

Zwart B, Ciurana C, Rensink I, Manoe R, Hack CE & Aarden LA (2004) Complement activation by apoptotic cells occurs predominantly via IgM and is limited to late apoptotic (secondary necrotic) cells. Autoimmunity 37(2): 95–102.

Appendix

Table 8. Primers used for amplification of V_H, V_κ, V_λ, C_H1, C_κ and C_λ.

Primer	Sequence
VH5´sense primers	
HFabVH1-F	5´GCTGCCCAACCAGCCATGGCCCAGGTGCAGCTGGTGCAGTCTGG3´
HFabVH2-F	5´GCTGCCCAACCAGCCATGGCCCAGATCACCTTGAAGGAGTCTGG3´
HFabVH35-F	5´GCTGCCCAACCAGCCATGGCCGAGGTGCAGCTGGTGSAGTCTGG3´
HFabH3a-F	5´GCTGCCCAACCAGCCATGGCCGAGGTGCAGCTGKTGGAGTCTG3´
HFabVH4-F	5´GCTGCCCAACCAGCCATGGCCCAGGTGCAGCTGCAGGAGTCGGG3´
HFabVH4a-F	5´GCTGCCCAACCAGCCATGGCCCAGGTGCAGCTACAGCAGTGGGG3´
VH3´reverse primers	
HFabVHJa-B	5´CGATGGGCCCTTGGTGGAGGCTGAGGAGACGGTGACCAGGGTTCC3´
HFabVHJb-B	5´CGATGGGCCCTTGGTGGAGGCWGRGGAGACGGTGACCAGGGTBCC3´
Vκ5´sense primers	
HSCK1-F	5´GGGCCCAGGCGGCCGAGCTCCAGATGACCCAGTCTCC3´
HSCK24-F	5´GGGCCCAGGCGGCCGAGCTCGTGATGACYCAGTCTCC3´
HSCK3-F	5´GGGCCCAGGCGGCCGAGCTCGTGWTGACRCAGTCTCC3´
HSCK5-F	5´GGGCCCAGGCGGCCGAGCTCACACTACGCAGTCTCC3´
Vκ3´reverse primer	
HCK5-B	5´GAAGACAGATGGTGCAGCCACAGT3´
Vλ5´sense primers	
HSCLam1a	5´GGGCCCAGGCGGCCGAGCTCGTGBTGACGCAGCCGCCCTC3´
HSCLab1b	5´GGGCCCAGGCGGCCGAGCTCGTGCTGACTCAGCCACCCTC3´
HSCLam2	5´GGGCCCAGGCGGCCGAGCTCGCCCTGACTCAGCCTCCCTCCGT3´
HSCLam3	5´GGGCCCAGGCGGCCGAGCTCGAGCTGACTCAGCCACCCTCAGTGTC3´
HSCLam4	5´GGGCCCAGGCGGCCGAGCTCGTGCTGACTCAATCGCCCTC3´
HSCLam6	5´GGGCCCAGGCGGCCGAGCTCATGCTGACTCAGCCCCACTC3´
HSCLam78	5´GGGCCCAGGCGGCCGAGCTCGTGGTGACYCAGGAGCCMTC3´
HSCLam9	5´GGGCCCAGGCGGCCGAGCTCGTGCTGACTCAGCCACCTTC3´
HSCLam10	5´GGGCCCAGGCGGCCGAGCTCGGGCAGACTCAGCAGCTCTC3´
Vλ3' reverse primer	
HL5-B	5´CGAGGGGGCAGCCTTGGGCTGACC3´
CH1 primers	
HIgGCH1-F (sense)	5´GCCTCCACCAAGGGCCCATCGGTC3´
dpseq (reverse)	5`AGAAGCGTAGTCCGGAACGTC3´
Cκ primers	
HKC-F (sense)	5´CGAACTGTGGCTGCACCATCTGTC3´
Lead-B (reverse)	5´GGCCATGGCTGGTTGGGCAGC3´
Cλ primers	
HLC-F (sense)	5´GGTCAGCCCAAGGCTGCCCCC3´
Lead-B reverse	5´GGCCATGGCTGGTTGGGCAGC3´

Table 9. Primers used for V_H/C_H and V_L/C_L overlap assembly and H/L assembly.

Primer	Sequence
V_H/C_H assembly	
LeadVH (sense)	5´GCTGCCCAACCAGCCATGGCC3´
dpseq (reverse)	5`AGAAGCGTAGTCCGGAACGTC3´
V_L/C_L assembly	
RSC-F (sense)	5´GAGGAGGAGGAGGAGGAGGCGGGGCCCAGGCGGCCGAGCTC3´
Lead B (reverse)	5´GGCCATGGCTGGTTGGGCAGC3´
H/L assembly	
RSC-F (sense)	5´GAGGAGGAGGAGGAGGAGGCGGGGCCCAGGCGGCCGAGCTC3´
dp-Ex (reverse)	5´GAGGAGGAGGAGGAGGAGAGAAGCGTAGCTCGGAACGTC3´

Original articles

I Kummu O, Turunen SP, Wang C, Lehtimäki J, Veneskoski M, Kastarinen H, Koivula MK, Risteli J, Kesäniemi YA & Hörkkö S (2013) Carbamyl adducts on low-density lipoprotein induce IgG response in $LDLR^{-/-}$ mice and bind plasma autoantibodies in humans under enhanced carbamylation. Antioxid Redox Signal 19(10): 1047–1062.

II Kummu O, Turunen SP, Prus P, Lehtimäki J, Veneskoski M, Wang C & Hörkkö S (2014) Human monoclonal Fab and human plasma antibodies to carbamyl-epitopes cross-react with malondialdehyde-adducts. Immunology 141(3): 416–430.

III Kummu O, Turunen SP, Harila K, Nissinen AET, Kastarinen H, Wang C & Hörkkö S (2014) Hemodialysis patients have plasma IgG autoantibodies binding to apoptotic cells. Manuscript.

1242. Syrjänen, Riikka (2014) TIM family molecules in hematopoiesis

1243. Kauppila, Joonas (2014) Toll-like receptor 9 in alimentary tract cancers

1244. Honkavuori-Toivola, Maria (2014) The prognostic role of matrix metalloproteinase-2 and -9 and their tissue inhibitor-1 and -2 in endometrial carcinoma

1245. Pienimäki, Tuula (2014) Factors, complications and health-related quality of life associated with diabetes mellitus developed after midlife in men

1246. Kallio, Miki (2014) Muuttuuko lääketieteen opiskelijoiden käsitys terveydestä peruskoulutuksen aikana : kuusivuotinen seurantatutkimus

1247. Haanpää, Maria (2014) Hereditary predisposition to breast cancer – with a focus on *AATF*, *MRG15*, *PALB2*, and three Fanconi anaemia genes

1248. Alanne, Sami (2014) Musiikkipsykoterapia : teoria ja käytäntö

1249. Nagy, Irina I. (2014) Wnt-11 signaling roles during heart and kidney development

1250. Prunskaite-Hyyryläinen, Renata (2014) Role of Wnt4 signaling in mammalian sex determination, ovariogenesis and female sex duct differentiation

1251. Huusko, Johanna (2014) Genetic background of spontaneous preterm birth and lung diseases in preterm infants : studies of potential susceptibility genes and polymorphisms

1252. Jämsä, Ulla (2014) Kuntoutuksen muutosagentit : tutkimus työelämälähtöisestä oppimisesta ylemmässä ammattikorkeakoulutuksessa

1253. Kaikkonen, Leena (2014) p38 mitogen-activated protein kinase and transcription factor GATA-4 in the regulation of cardiomyocyte function

1254. Finnilä, Mikko A. J. (2014) Bone toxicity of persistent organic pollutants

1255. Starck, Tuomo (2014) Dimensionality, noise separation and full frequency band perspectives of ICA in resting state fMRI : investigations into ICA in resting state fMRI

1256. Karhu, Jaana (2014) Severe community- acquired pneumonia – studies on imaging, etiology, treatment, and outcome among intensive care patients

1257. Lahti, Anniina (2014) Epidemiological study on trends and characteristics of suicide among children and adolescents in Finland

1258. Nyyssönen, Virva (2014) Transvaginal mesh-augmented procedures in gynecology : outcomes after female urinary incontinence and pelvic organ prolapse surgery

Book orders:
Granum: Virtual book store
http://granum.uta.fi/granum/

UNIVERSITY OF OULU P.O. Box 8000 FI-90014 UNIVERSITY OF OULU FINLAND

ACTA UNIVERSITATIS OULUENSIS

ISBN 978-952-62-0566-3 (Paperback)
ISBN 978-952-62-0567-0 (PDF)
ISSN 0355-3221 (Print)
ISSN 1796-2234 (Online)

UNIVERSITY of OULU
OULUN YLIOPISTO