FUTURE DRUGS IN ATHEROSCLEROTIC CARDIOVASCULAR DISEASE

EVELINE VAN POELGEEST

VOOR MIJN OUDERS

COVER PICTURE

The Doctor, Gerrit Dou *(copy after)*, 1650 - 1669
painter: Jan Adriaensz. van Staveren
(both belonging to the group of 'Leidse Fijnschilders')
oil on copper, 49cm × 37cm
Rijksmuseum, Amsterdam, the Netherlands

FUNDING

Publication of this thesis was financially supported by the foundation
Centre for Human Drug Research, Leiden, the Netherlands

DESIGN

Caroline de Lint, Voorburg (caro@delint.nl)

FUTURE DRUGS IN ATHEROSCLEROTIC CARDIOVASCULAR DISEASE

PROEFSCHRIFT
ter verkrijging van de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties,
te verdedigen op 10 april 2018 klokke 16:15 uur

DOOR
Eveline Petra van Poelgeest
geboren te Almelo
in 1978

PROMOTORES

Prof. dr. J. Burggraaf

Prof. dr. A.F. Cohen

CO-PROMOTOR

Dr. M. Moerland (CHDR)

LEDEN PROMOTIECOMMISSIE

Prof. dr. H.C.J. Eikenboom

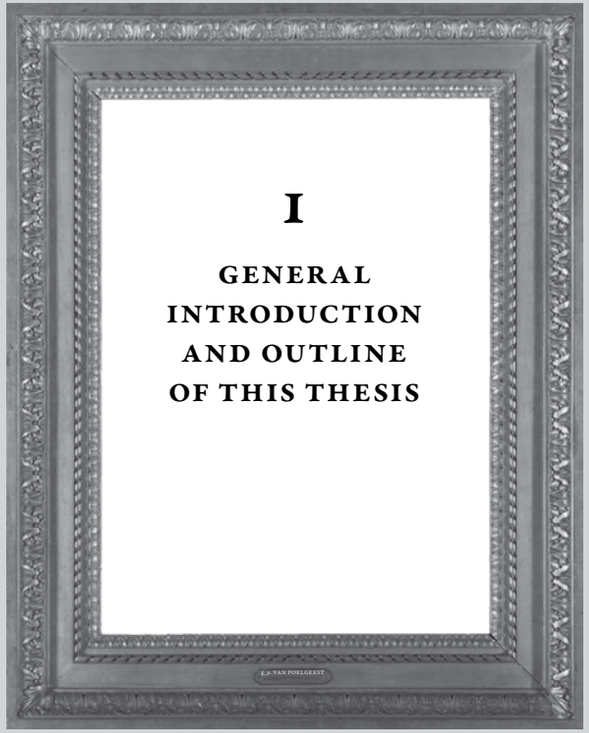
Prof. dr. J.W. de Fijter

Prof. dr. H.A.H. Kaasjager

Prof. dr. S. Middeldorp

.....

Dr. N. van der Velde



I

GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS

Atherosclerosis is a chronic disease of medium-sized and large arteries [1], caused by increased levels of low-density lipoprotein cholesterol (LDL-C), the principal atherogenic lipoprotein in the blood that promotes cholesterol accumulation and a subsequent inflammatory response within the artery wall characterized by impaired endothelial cell homeostasis [2-4].

CURRENT CARDIOVASCULAR PHARMACOTHERAPY, AIMS OF THIS THESIS

The most important risk factor for atherosclerotic cardiovascular disease (ACVD) is increased levels of LDL-C. Therefore, international guidelines uniformly recommend aggressive LDL-C lowering in patients who are at risk for ACVD [5;6]. Statins (HMG-coenzyme A reductase inhibitors) have long been the most potent LDL-C lowering drugs on the market. Not surprisingly, they have been the standard of care in ACVD risk reduction. However, statin treatment is complicated by the fact that a considerable number of patients is unable to tolerate full therapeutic doses due to adverse effects [7], or can be classified as statin low or non-responders (<10% reduction in LDL-C) [4;8;9]. In >25% of patients at (very) high risk for cardiovascular disease, statin efficacy is too limited to achieve current guideline-mandated LDL-C target goals [10], and aggressive statin therapy decreases relative risk for ASCD by only 30-35% [11], leaving an unacceptable relative risk of 65-70% for life-threatening events [12], referred to as 'residual risk' in clinical practice [13]. From large-scale clinical studies [14;15] it is clear that this risk is determined equally by on-treatment LDL-C levels and on-treatment measures of systemic inflammation: half of these patients have low systemic inflammatory burden but high levels of cholesterol (residual cholesterol risk), and therefore would benefit from additional cholesterol lowering drugs. The remainder has adequately low cholesterol levels but an increased inflammatory burden (residual inflammatory risk), and would benefit from treatments that lower inflammation. In both pertaining patient categories, effective therapy has been lacking for decades. Thus, there is an urgent unmet clinical need for reducing residual risk in atherosclerosis with novel drugs that counteract the key pathophysiologic elements of atherosclerosis, namely: (1) increased LDL-C levels, (2) inflammation, and (3) dysfunctional endothelial barrier function resulting in subendothelial cholesterol accumulation and subsequent atheroma formation. In this thesis, we describe the first clinical studies with novel compounds based on themes 1 and 2 (including the required methodology) and present the methodology that may be useful to develop future compounds based on theme 3.

EVOLVING APPROACHES IN CARDIOVASCULAR PHARMACOTHERAPY

Novel non-statin approaches to reduce cardiovascular morbidity and mortality are under evaluation in basic preclinical investigations and clinical trials. Thematically organized, these approaches include but are not limited to: (1) increasing serum LDL-C clearance through modulation of LDL-receptor (LDL-R) expression [16-18], (2) selective non-statin based inhibition of systemic low-grade inflammation blocking crucial proinflammatory cytokines [19;20], and (3) ameliorate endothelial dysfunction by decreasing (non-LDL related high rates of) subendothelial cholesterol accumulation [21;22]. Results from large scale prospective clinical (outcome) studies on the first 2 themes are expected to be published in the course of 2018. They are expected to finally confirm the 'even lower is even better' LDL-C hypothesis, and inflammation theory in atherosclerosis pathophysiology, respectively, paving the way for a revolution in clinical atherosclerotic cardiovascular pharmacology.

TARGETING RESIDUAL LIPID RISK, PCSK9 INHIBITION

The identification of mutations in the proprotein convertase subtilisin/kexin type 9 (PCSK9) gene causing dominant hypercholesterolemia [23] in 2003 led to an exciting breakthrough in the field of cardiovascular pharmacology. PCSK9 is a secreted glycoprotein that transcriptionally regulates cholesterol homeostasis. The enzyme promotes lysosomal degradation of hepatocyte LDL-Rs in hypercholesterolemia. Gain-of-function mutations in the PCSK9 gene leads to decreased numbers of LDL-Rs and consequently increased LDL-C levels and premature cardiovascular disease; loss-of-function mutations are associated with lifelong reduced levels of LDL-C, and a nearly 50% lower risk of coronary heart disease [24]. Inhibition of the enzyme increases LDL-Rs on the hepatocyte cell surface, and thereby increased clearance of LDL-C from the circulation. Importantly, statin treatment increases PCSK9 levels through negative feedback, thus promoting LDL-R degradation and limiting statin LDL-C lowering capacity [25]. Furthermore, genetic PCSK9 variations may be involved in causing high inter-individual variability (5-70%) in statin-induced LDL-C reduction [7]. Interestingly, recent observations suggest that PCSK9 has non-lipid anti-inflammatory effects, blunting atherogenesis by alleviating endothelial dysfunction and inflammation of the vessel wall [26-30]. Taken together, these data show that PCSK9 inhibition is a promising pharmacological intervention to reduce residual cholesterol risk [31], and possibly residual inflammatory risk, both in patients with and without statin therapy. In **CHAPTER 2**,

we describe how we targeted excess LDL-C with SPC5001, a novel antisense oligonucleotide (ASO) directed against PCSK9 in healthy volunteers with elevated LDL-C levels (Figure 1). ASOs are short, synthetic oligonucleotide analogues designed to bind directly to specific RNAs through Watson-Crick base pairing. These compounds exert their pharmacological effect by high-specificity interference with gene transcription after hybridizing to target RNA, ultimately resulting in inhibition of intra- and extracellular synthesis of a specific protein [31]. Because ASOs accumulate in the kidney and PCSK9 expression is pronounced in the kidney [32], kidney function was meticulously evaluated to make sure that SPC5001-like other ASOs, and in line with extensive preclinical toxicology testing- had no toxic effects on the kidney. Unfortunately, SPC5001 appeared to negatively affect kidney function in our clinical study. Generally accepted biomarkers (e.g. serum creatinine and blood urea nitrogen) lack sensitivity and fail to detect early subtle signs of acute kidney injury (AKI), while the extent of injury and poor outcomes associated with AKI worsen with delayed recognition of impending injury. Thus, there is an urgent need to identify novel kidney injury markers that detect (subtle) signs of cellular injury, and offer guidance in clinical decision making. Upon the first signs of renal toxicity of SPC5001, we retrospectively measured a panel of promising novel biomarkers for their potential to capture subtle signs of injury earlier than the markers currently employed in clinical practice [33]. In **CHAPTER 3** we discuss whether these novel kidney injury biomarkers may be of benefit for future renal toxicology screening programs.

TARGETING RESIDUAL INFLAMMATION RISK, TLR4 SIGNALING BLOCKADE

(Pre-) clinical data collected in the past four decades convincingly demonstrate that inflammation is the driving force behind all pathophysiological phases of atherosclerotic disease [34;35]. It is well established that statins reduce cardiovascular risk partly through cholesterol-independent immunoregulatory and anti-inflammatory pleiotropic effects: they improve endothelial function and plaque stabilization and decrease vascular inflammation [36;37]. Statin-related anti-inflammatory effect size, however, is only limited: one-third of patients on statin treatment have high levels of inflammation despite adequate cholesterol levels (residual inflammatory risk for (recurrent) atherosclerotic cardiovascular events) [14;15]. The question whether modulation of systemic inflammation per se (i.e. without concomitant cholesterol lowering and/or platelet aggregation) is effective in preventing events, however, remains unanswered [38]. Most likely, this is explained by the highly complex pathophysiology of both cholesterol metabolism and immune (counter) regulatory pathways, which operate in cross-talk in atherosclerosis [4]. Until now, most immunoregulatory interventions have focused on reducing CRP. Although the role of this downstream inflammatory biomarker is well established in cardiovascular risk prediction [39;40], clinical trials have failed to show that pharmacological targeting of CRP reduces cardiovascular risk [34]. Interfering further upstream in the inflammatory cascades resulting in reduced IL1β and/or IL6 production may be a more successful approach [34;41;42], since these proatherogenic cytokines play key roles in the core of atherosclerosis development [41]. Upstream in the pathophysiologic inflammatory cascade in atherosclerosis Toll-like receptor 4 (TLR4) plays an important role (Figure 2). Ligands for TLR4 signaling are lipopolysaccharide (LPS) and (modified) LDL; excessive or prolonged LPS induced TLR4 signaling in effector cells such as macrophages and endothelial cells has been associated with (amplification of) chronic systemic low-grade inflammation, leading to endothelial dysfunction and subsequent cardiovascular disease [43;44]. In the presence of cholesterol crystals TLR4 signaling may also lead to NOD-, LRR- and pyrin domaincontaining 3 (NLRP3) inflammasome activation. Inflammasomes have been shown to be intracellular pattern recognition complexes of proteins involved in the maturation and secretion of IL1β in complex chronic diseases such as atherosclerosis and type 2 diabetes mellitus [45]. Unbalanced TLR and subsequent inflammasome signaling disrupt counter-regulatory LDL clearance mechanisms, causing perpetuation and amplification of inflammatory signaling. This apparent preference for innate immunity at the expense of cholesterol clearance likely causes (accelerated) atherogenesis in chronic inflammatory conditions including obesity, metabolic syndrome, and type 2 diabetes mellitus [46]. Considering the global epidemic of these conditions, detailed insight into involved inflammatory signaling and pharmacological inhibition thereof is of great importance. Clearly, pharmacological inhibition of TLR4 signaling may be an effective approach for inflammation-induced (accelerated) atherogenesis.

Novimmune developed NI-0101, a monoclonal antibody blocking TLR4 signaling for blunting systemic inflammation. In order to evaluate the drugs intended pharmacology in healthy volunteers, a TLR4 challenge test was applied: the human endotoxemia model is a well-established model for studying inflammation and anti-inflammatory signaling pathways in preclinical drug development. In this experimental setting, LPS (a constituent of the outer membrane of Gram-negative bacteria) is intravenously administered to healthy volunteers to induce systemic inflammation through TLR4 signaling. The commonly applied relatively

high LPS dose (2-4 ng/kg bodyweight), however, is unnecessarily noxious, and induces an overshoot in the immune response that impedes evaluation of potential effects of immune-modulating interventions in chronic low-grade inflammatory cardio metabolic conditions such as atherosclerotic disease. Low-dose (1 ng/kg) experimental endotoxemia induces inflammatory and metabolic changes that closely resemble those observed in these conditions [47;48]. Thorough characterization of inflammatory effects of low-dose endotoxemia is therefore desired. The aim of **CHAPTER 4** was to characterize the inflammatory effects of low-grade endotoxemia. To this end, we administrated (very) low-dose (0.5, 1 and 2 ng/kg) LPS intravenously (*in vivo* endotoxemia; Figure 3A) and in whole blood (*ex vivo* endotoxemia model; Figure 3B). We explored whether the inflammatory effects of *ex vivo* whole blood LPS challenging are well comparable with the *in vivo* LPS challenge. If *ex vivo* testing appears a reliable surrogate of *in vivo* testing, this would improve and simplify future pharmacology studies. Compared to *in vivo* testing, *ex vivo* testing is less invasive and more convenient (*ex vivo* testing can be repeated over time in the same person).

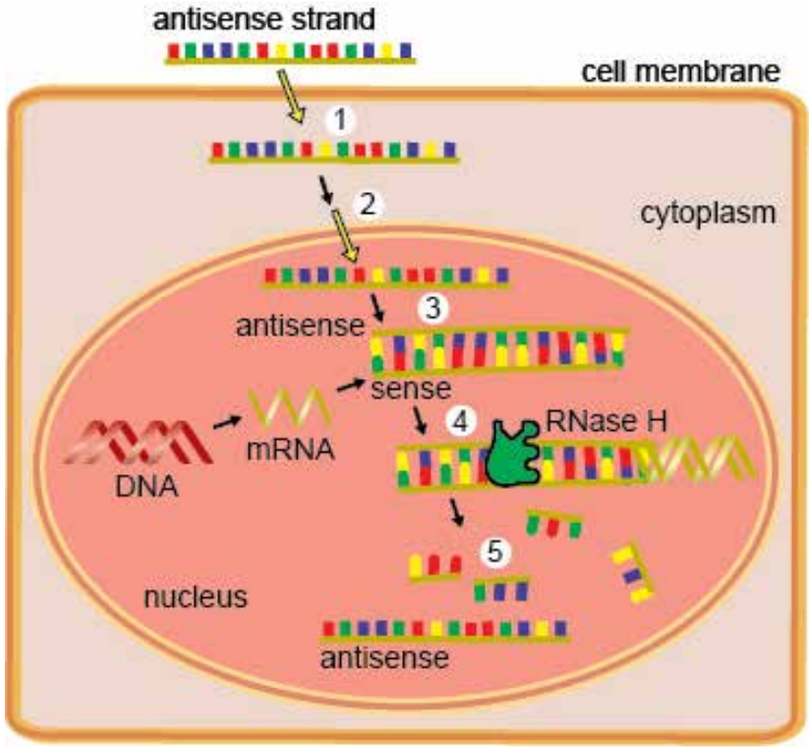
The results of our TLR4 challenge test guided the design of our clinical trial described in **CHAPTER 5**, in which we explored the anti-inflammatory potential of NI-0101 in *in vivo* and *ex vivo* LPS challenge tests (Figure 3C).

TARGETING VASCULAR DYSFUNCTION

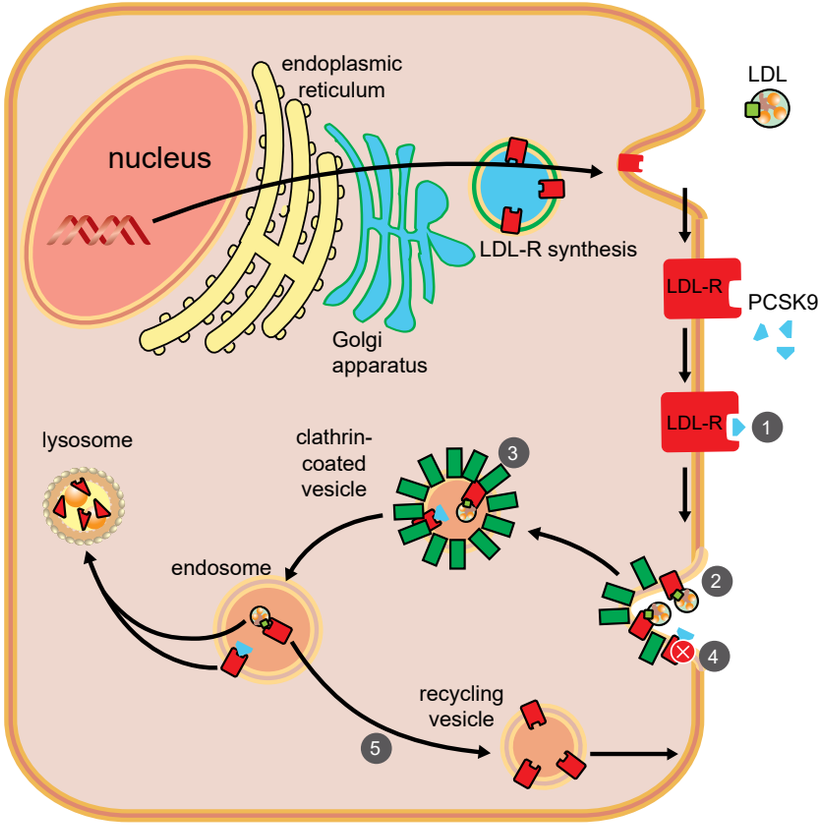
Pharmacological interventions targeting endothelial activation/dysfunction may be an interesting approach in ACVD because it links hypercholesterolemia and inflammation, two key players in the pathophysiology of atherosclerotic disease. Healthy endothelial cells effectively maintain vascular wall homeostasis. Increased levels of LDL-C activate endothelial cells, shifting their physiologically anti-atherothrombotic features into pathophysiological pro-atherothrombotic features [49]. This systemic condition of endothelial activation, called endothelial dysfunction, is critical in the pathogenesis of atherosclerosis [49-51]: increased LDL levels cause faulty endothelial permeability which allows cholesterol-laden low density lipoprotein particles to migrate into the intima of the arterial vessel wall. Subendothelial LDL accumulation is prone to modification (e.g. to minimally modified LDL and oxidized LDL [3]), aggregation and formation of cholesterol crystals [52], triggering a proatherogenic inflammatory response initiated by attracting monocytes to the lesion site [53]. Monocytes subsequently differentiate into macrophages which take up the modified lipoproteins and become characteristic foam cells. Foam cells in turn release a variety of proinflammatory cytokines [42] and accumulate into fatty streaks that further stimulate the inflammatory process to ultimately mature into atherosclerotic plaques. Relatively high-dose (4 ng/kg bodyweight) endotoxin exposure is associated with endothelial activation/dysfunction [54;55] and kidney injury [56]. Systematically collected quantitative and temporal data on low-dose endotoxin-induced activation of the human microvasculature and/or (subclinical) kidney injury are not readily available in the public domain. Therefore, we characterized the effects of low-dose LPS on the endothelium, and explored whether the low-dose *in vivo* endotoxin model could also qualify for broader application in clinical development of future drugs designed to protect endothelial integrity. These investigations are described in **CHAPTER 6**.

Finally, in **CHAPTER 7** all results obtained in this thesis and their implications are summarized and discussed.

Figure 1. LDL-C lowering by SPC5001, an antisense oligonucleotide directed against PCSK9

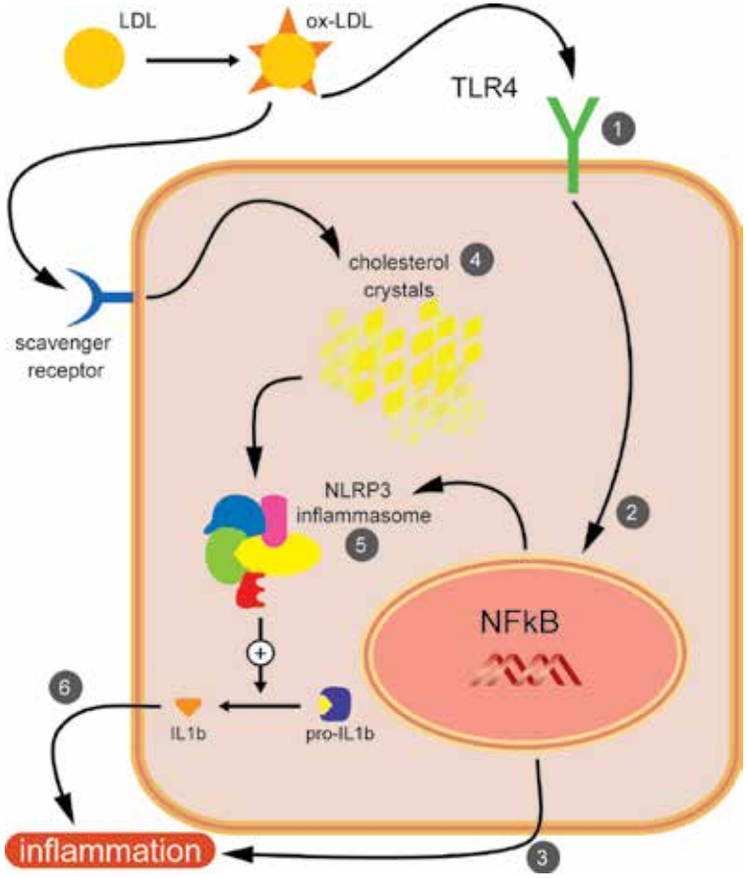


PANEL A. ANTISENSE OLIGONUCLEOTIDE MECHANISM OF ACTION. Single-stranded oligonucleotides are transported across the plasma membrane (step 1). In the cytoplasm, single-stranded oligonucleotides rapidly accumulate in the nucleus (steps 2 and 3), where they bind to their targeted RNA (step 4). Once bound to the RNA, RNAase H recognizes the oligonucleotide (RNA duplex) as a substrate, cleaving the RNA strand and releasing the antisense oligonucleotide (step 5). The cleavage occurs predominantly in the nucleus, but also in the cytosol.



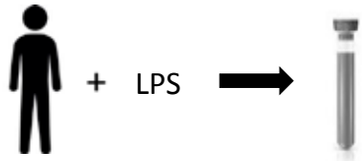
PANEL B. PCSK9 MECHANISM OF ACTION, AND PCSK9 INHIBITION BY SPC5001. PCSK9 binds to the LDL-R, locking it to an open configuration (step 1); the resulting complex is transported from the cell membrane into the cell by clathrin-mediated endocytosis (step 2); the open configuration of the LDL-R directs the complex towards lysosomal degradation (step 3). Besides endocytosis-mediated LDL-R degradation, PCSK9 directly acts intracellularly to enhance LDL-R degradation (not shown). LDL-R degradation prevents the LDL-R to be recycled, resulting in LDL accumulation and subsequent modification (e.g. oxidized LDL), ultimately leading to foam cell and atheroma formation. SPC5001 inhibits PCSK9 production and secretion, precluding it from binding to the LDL-R (step 4) and preventing LDL-R degradation intracellularly (not shown). Instead, LDL-C binds to its receptor, leading to internalization of the complex and subsequent degradation leading to recycling of the LDL-R to the cell surface (step 5), facilitating clearance of serum LDL-C. Moreover, atherogenic Apolipoprotein B is degraded, and cholesterol formed for maintenance of cell function (not shown).

Figure 2. TLR4 signaling and inflammasome activation.

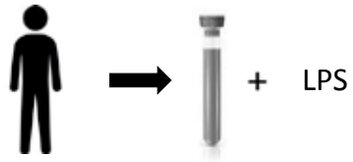


Both exogenous (e.g. lipopolysaccharides) and endogenous (e.g. oxidized LDL-C) ligands can ligate TLR4 (step 1) on cells such as macrophages, vascular smooth muscle cells, dendritic cells and endothelial cells. Ligand binding activates the myeloid differentiation primary response protein 88 (MYD88)-dependent and TIR domain-containing Adaptor inducing IFNβ (TRIF) (or MYD88-independent) pathways (step 2) leading to NFkB related release of proatherogenic inflammatory cytokines (e.g. TNFα, IL6), chemokines (e.g. CXCL10), cell adhesion molecules (e.g. ICAM1, VCAM1), selectins (e.g. E-selectin), proteases and reactive oxygen species (step 3). Also, intracellular cholesterol crystals (4) can exert proatherogenic effects by stimulating IL1β production by macrophages through NLRP3 inflammasome activation (step 5), leading to additional inflammatory responses (step 6).

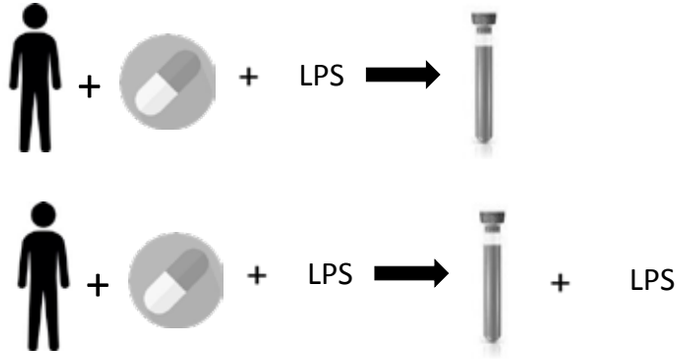
Figure 3. Schematic representation of the methodological and pharmacological interventions applied in this thesis.



IN VIVO LPS CHALLENGE TEST (CHAPTERS 4 AND 6).
In the *in vivo* LPS challenge test, healthy volunteers are administered lipopolysaccharide intravenously to induce a transient state of systemic inflammation.



EX VIVO LPS CHALLENGE TEST (CHAPTERS 4 AND 6).
In the *ex vivo* LPS challenge test, lipopolysaccharide is added to whole blood samples from healthy volunteers.

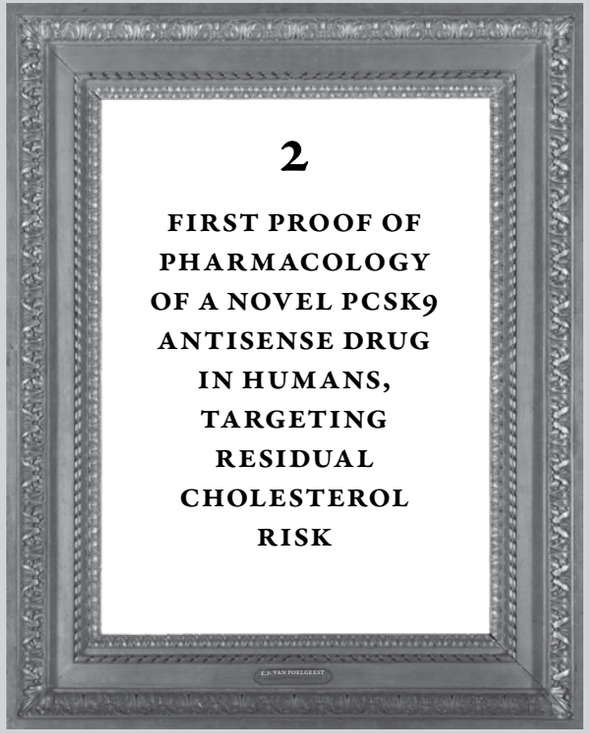


N VIVO (UPPER) AND COMBINED IN VIVO/EX VIVO (LOWER) LPS CHALLENGE TEST IN EVALUATION OF THE ANTI-INFLAMMATORY EFFECTS OF TLR4 INHIBITOR NI-0101 (CHAPTER 5).

REFERENCES

1 Falk E. Pathogenesis of atherosclerosis. J Am Coll Cardiol 2006; 47: C7-12.
2 Schaftenaar F, Frodermann V, Kuiper J, Lutgens E. Atherosclerosis: the interplay between lipids and immune cells. Curr Opin Lipidol. 2016; 27: 209-215.
3 Glass CK, Witztum JL. Atherosclerosis. the road ahead. Cell 2001; 104: 503-516.
4 Tall AR, Yvan-Charvet L. Cholesterol, inflammation and innate immunity. Nat Rev Immunol 2015; 15: 104-116.
5 Piepoli MF, Hoes AW, Agewall S, Albus C, Brotons C, Catapano AL, Cooney MT, Corra U, Cosyns B, Deaton C, Graham I, Hall MS, Hobbs FD, Lochen ML, Lollgen H, Marques-Vidal P, Perk J, Prescott E, Redon J, Richter DJ, Sattar N, Smulders Y, Tiberi M, van der Worp HB, van D, I, Verschuren WM. 2016 european Guidelines on cardiovascular disease prevention in clinical practice: The Sixth Joint Task Force of the european Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of 10 societies and by invited experts) Developed with the special contribution of the european Association for Cardiovascular Prevention & Rehabilitation (EACPR). eur Heart J 2016; 37: 2315-2381.
6 Stone NJ, Robinson JG, Lichtenstein AH, Bairey Merz CN, Blum CB, Eckel RH, Goldberg AC, Gordon D, Levy D, Lloyd-Jones DM, McBride P, Schwartz JS, Shero ST, Smith SC, Jr., Watson K, Wilson PW, Eddleman KM, Jarrett NM, LaBresh K, Nevo L, Wnek J, Anderson JL, Halperin JL, Albert NM, Bozkurt B, Brindis RG, Curtis LH, DeMets D, Hochman JS, Kovacs RJ, Ohman EM, Pressler SJ, Sellke FW, Shen WK, Smith SC, Jr., Tomaselli GF. 2013 ACC/AHA guideline on the treatment of blood cholesterol to reduce atherosclerotic cardiovascular risk in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. circulation 2014; 129: S1-45.
7 Reiner Z. Resistance and intolerance to statins. Nutr Metab Cardiovasc.Dis 2014; 24: 1057-1066.
8 Feng Q, Wei WQ, Chung CP, Levinson RT, Bastarache L, Denny JC, Stein CM. The effect of genetic variation in PCSK9 on the LDL-Cholesterol response to statin therapy. Pharmacogenomics J 2016.
9 Taylor BA, Thompson PD. Statins and Their Effect on PCSK9-Impact and Clinical Relevance. Curr Atheroscler.Rep 2016; 18: 46.
10 Nissen SE, Nicholls SJ, Sipahi I, Libby P, raichlen JS, Ballantyne CM, Davignon J, Erbel R, Fruchart JC, Tardif JC, Schoenhagen P, Crowe T, Cain V, Wolski K, Goormastic M, Tuzcu EM. Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: the ASTEROID trial. JAMA 2006; 295: 1556-1565.
11 Law MR, Wald NJ, Rudnicka AR. Quantifying effect of statins on low density lipoprotein cholesterol, ischAEmic heart disease, and stroke: systematic review and meta-analysis. BMJ 2003; 326: 1423.
12 Libby P, Bornfeldt KE, Tall AR. Atherosclerosis: Successes, Surprises, and Future Challenges. circ.Res 2016; 118: 531-534.
13 Ridker PM. Residual inflammatory risk: addressing the obverse side of the atherosclerosis prevention coin. eur Heart J 2016; 37: 1720-1722.
14 Bohula EA, Giugliano RP, Cannon CP, Zhou J, Murphy SA, White JA, Tershakovec AM, Blazing MA, Braunwald E. Achievement of dual low-density lipoprotein cholesterol and high-sensitivity C-reactive protein targets more frequent with the addition of ezetimibe to simvastatin and associated with better outcomes in IMPROVE-IT. circulation 2015; 132: 1224-1233.
15 Ridker PM, Cannon CP, Morrow D, Rifai N, Rose LM, McCabe CH, Pfeffer MA, Braunwald E. C-reactive protein levels and outcomes after statin therapy. N Engl J Med 2005; 352: 20-28.
16 Cannon CP, Cariou B, Blom D, McKenney JM, Lorenzato C, Pordy R, Chaudhari U, Colhoun HM. Efficacy and safety of alirocumab in high cardiovascular risk patients with inadequately controlled hypercholesterolAEmia on maximally tolerated doses of statins: the ODYSSEY COMBO II randomized controlled trial. eur Heart J 2015; 36: 1186-1194.
17 Koren MJ, Scott R, Kim JB, Knusel B, Liu T, Lei L, Bolognese M, Wasserman SM. Efficacy, safety, and tolerability of a monoclonal antibody to proprotein convertase subtilisin/kexin type 9 as monotherapy in patients with hypercholesterolaemia (MENDEL): a randomised, double-blind, placebo-controlled, phase 2 study. Lancet 2012; 380: 1995-2006.
18 Feinberg MW, Moore KJ. MicroRNA Regulation of Atherosclerosis. circ.Res 2016; 118: 703-720.
19 Everett BM, Pradhan AD, Solomon DH, Paynter N, Macfadyen J, Zaharris E, Gupta M, Clearfield M, Libby P, Hasan AA, Glynn RJ, Ridker PM. rationale and design of the Cardiovascular Inflammation Reduction Trial: a test of the inflammatory hypothesis of atherothrombosis. Am Heart J 2013; 166: 199-207.
20 Ridker PM, Thuren T, Zalewski A, Libby P. Interleukin-1beta inhibition and the prevention of recurrent cardiovascular events: rationale and design of the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS). Am Heart J 2011; 162: 597-605.
21 KraEhling JR, Chidlow JH, rajagopal C, Sugiyama MG, Fowler JW, Lee MY, Zhang X, ramirez CM, Park EJ, Tao B, Chen K, Kuruvilla L, Larrivee B, Folta-Stogniew E, Ola R, Rotllan N, Zhou W, Nagle MW, Herz J, Williams KJ, Eichmann A, Lee WL, Fernandez-Hernando C, Sessa WC. Genome-wide RNAi screen reveals ALK1 mediates LDL uptake and transcytosis in endothelial cells. Nat Commun. 2016; 7: 13516.
22 Takase S, Matoba T, Nakashiro S, Mukai Y, Inoue S, Oi K, Higo T, Katsuki S, Takemoto M, Suematsu N, Eshima K, Miyata K, Yamamoto M, Usui M, SAdamatsu K, Satoh S, Kadokami T, Hironaga K, Ichi I, Todaka K, Kishimoto J, Egashira K, Sunagawa K. Ezetimibe in Combination With Statins Ameliorates Endothelial Dysfunction in Coronary Arteries After Stenting: The CUVIC Trial (Effect of Cholesterol Absorption Inhibitor USAge on Target Vessel Dysfunction After Coronary Stenting), a Multicenter randomized Controlled Trial. Arterioscler.Thromb Vasc Biol 2016.
23 Abifadel M, Varret M, rabes JP, Allard D, Ouguerram K, Devillers M, Cruaud C, Benjannet S, Wickham L, Erlich D, Derre A, Villeger L, Farnier M, Beucler I, Bruckert E, Chambaz J, Chanu B, Lecerf JM, Luc G, Moulin P, Weissenbach J, Prat A, Krempf M, Junien C, Seidah NG, Boileau C. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat Genet. 2003; 34: 154-156.
24 Cohen JC, Boerwinkle E, Mosley TH, Jr., Hobbs HH. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. N Engl J Med 2006; 354: 1264-1272.
25 Dadu RT, Ballantyne CM. Lipid lowering with PCSK9 inhibitors. Nat Rev Cardiol 2014; 11: 563-575.
26 Kuhnast S, van der Hoorn JW, Pieterman EJ, van den Hoek AM, SASiela WJ, Gusarova V, Peyman A, Schafer HL, Schwahn U, Jukema JW, Princen HM. Alirocumab inhibits atherosclerosis, improves the plaque morphology, and enhances the effects of a statin. J Lipid Res 2014; 55: 2103-2112.
27 Ferri N, Tibolla G, Pirillo A, cipollone F, Mezzetti A, Pacia S, Corsini A, Catapano AL. Proprotein convertase subtilisin kexin type 9 (PCSK9) secreted by cultured smooth muscle cells reduces macrophages LDL-R levels. Atherosclerosis 2012; 220: 381-386.
28 Giugliano RP, Sabatine MS. Are PCSK9 Inhibitors the Next Breakthrough in the Cardiovascular Field? J Am Coll Cardiol 2015; 65: 2638-2651.
29 Lan H, Pang L, Smith MM, Levitan D, Ding W, Liu L, Shan L, Shah VV, Laverty M, Arreaza G, Zhang Q, Murgolo NJ, Hernandez M, Greene JR, Gustafson EL, Bayne ML, Davis HR, Hedrick JA. Proprotein convertase subtilisin/kexin type 9 (PCSK9) affects gene expression pathways beyond cholesterol metabolism in liver cells. J Cell Physiol 2010; 224: 273-281.
30 Cheng JM, Oemrawsingh RM, Garcia-Garcia HM, Boersma E, van Geuns RJ, Serruys PW, Kardys I, Akkerhuis KM. PCSK9 in relation to coronary plaque inflammation: Results of the ATHEROREMO-IVUS study. Atherosclerosis 2016; 248: 117-122.
31 Bergeron N, Phan BA, Ding Y, Fong A, Krauss RM. Proprotein convertase subtilisin/kexin type 9 inhibition: a new therapeutic mechanism for reducing cardiovascular disease risk. circulation 2015; 132: 1648-1666.
32 Seidah NG, Benjannet S, Wickham L, Marcinkiewicz J, Jasmin SB, Stifani S, Basak A, Prat A, Chretien M. The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation. Proc Natl Acad Sci U S A 2003; 100: 928-933.
33 van Meer L, Moerland M, Cohen AF, Burggraaf J. Urinary kidney biomarkers for early detection of nephrotoxicity in clinical drug development. Br J Clin Pharmacol 2014; 77: 947-957.
34 Ridker PM. From C-Reactive Protein to Interleukin-6 to Interleukin-1: Moving Upstream To Identify Novel Targets for Atheroprotection. circ.Res 2016; 118: 145-156.
35 Libby P. Inflammation in atherosclerosis. Nature 2002; 420: 868-874.
36 Ito MK, Talbert RL, Tsimikas S. Statin-associated pleiotropy: possible beneficial effects beyond cholesterol reduction. Pharmacotherapy 2006; 26: 85S-97S.
37 Lahera V, Goicoechea M, de Vinuesa SG, Miana M, de las HN, Cachofeiro V, Luno J. Endothelial dysfunction, oxidative stress and inflammation in atherosclerosis: beneficial effects of statins. Curr Med Chem 2007; 14: 243-248.
38 Lorenzatti AJ, Retzlaff BM. Unmet needs in the management of atherosclerotic cardiovascular disease: Is there a role for emerging anti-inflammatory interventions? Int J Cardiol 2016; 221: 581-586.
39 Ridker PM, Buring JE, Shih J, Matias M, Hennekens CH. Prospective study of C-reactive protein and the risk of future cardiovascular events among apparently healthy women. circulation 1998; 98: 731-733.
40 Willerson JT, Ridker PM. Inflammation as a cardiovascular risk factor. circulation 2004; 109: II2-10.

41 Ridker PM, Luscher TF. Anti-inflammatory therapies for cardiovascular disease. Eur Heart J 2014; 35: 1782-1791.
42 Golia E, Limongelli G, Natale F, Fimiani F, Maddaloni V, Pariggiano I, Bianchi R, Crisci M, D'Acierno L, Giordano R, Di PG, Conte M, Golino P, Russo MG, Calabro R, Calabro P. Inflammation and cardiovascular disease: from pathogenesis to therapeutic target. Curr Atheroscler.Rep 2014; 16: 435.
43 Goulopoulou S, McCarthy CG, Webb RC. Toll-like Receptors in the Vascular System: Sensing the Dangers Within. Pharmacol Rev 2016; 68: 142-167.
44 Tsoyi K, Kim WS, Kim YM, Kim HJ, Seo HG, Lee JH, Yun-Choi HS, Chang KC. Upregulation of ptEN by CKD712, a synthetic tetrahydroisoquinoline alkaloid, selectively inhibits lipopolysaccharide-induced VCAM-1 but not ICAM-1 expression in human endothelial cells. Atherosclerosis 2009; 207: 412-419.
45 De ND, Latz E. NLRP3 inflammasomes link inflammation and metabolic disease. Trends Immunol 2011; 32: 373-379.
46 McGillicuddy FC, de la Llera MM, Hinkle CC, Joshi MR, Chiquoine EH, Billheimer JT, Rothblat GH, Reilly MP. Inflammation impairs reverse cholesterol transport in vivo. circulation 2009; 119: 1135-1145.
47 Patel PN, Shah RY, Ferguson JF, Reilly MP. Human experimental endotoxemia in modeling the pathophysiology, genomics, and therapeutics of innate immunity in complex cardiometabolic diseases. Arterioscler.Thromb Vasc Biol 2015; 35: 525-534.
48 de la Llera MM, McGillicuddy FC, Hinkle CC, Byrne M, Joshi MR, Nguyen V, Tabita-Martinez J, Wolfe ML, Badellino K, Pruscino L, Mehta NN, Asztalos BF, Reilly MP. Inflammation modulates human HDL composition and function in vivo. Atherosclerosis 2012; 222: 390-394.
49 Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk. Arterioscler.Thromb Vasc Biol 2003; 23: 168-175.
50 Ross R. Atherosclerosis–an inflammatory disease. N Engl J Med 1999; 340: 115-126.
51 Sitia S, Tomasoni L, Atzeni F, Ambrosio G, Cordiano C, Catapano A, Tramontana S, Perticone F, Naccarato P, Camici P, Picano E, Cortigiani L, Bevilacqua M, Milazzo L, Cusi D, Barlassina C, Sarzi-Puttini P, Turiel M. From endothelial dysfunction to atherosclerosis. Autoimmun.Rev 2010; 9: 830-834.
52 Hansson GK, Hermansson A. The immune system in atherosclerosis. Nat Immunol 2011; 12: 204-212.
53 Ghattas A, Griffiths HR, Devitt A, Lip GY, Shantsila E. Monocytes in coronary artery disease and atherosclerosis: where are we now? J Am Coll Cardiol 2013; 62: 1541-1551.
54 Jilma B, Blann A, Pernerstorfer T, Stohlawetz P, Eichler HG, Vondrovec B, Amiral J, Richter V, Wagner OF. Regulation of adhesion molecules during human endotoxemia. No acute effects of aspirin. Am J Respir Crit Care Med 1999; 159: 857-863.
55 Fijen JW, Tulleken JE, Kobold AC, de BP, van der Werf TS, Ligtenberg JJ, Spanjersberg R, Zijlstra JG. Inhibition of p38 mitogen-activated protein kinase: dose-dependent suppression of leukocyte and endothelial response after endotoxin challenge in humans. Crit Care Med 2002; 30: 841-845.
56 Zijlstra JG, Tulleken JE, Ligtenberg JJ, de BP, van der Werf TS. p38-MAPK inhibition and endotoxin induced tubular dysfunction in men. J Endotoxin.Res 2004; 10: 402-405.



2

FIRST PROOF OF PHARMACOLOGY OF A NOVEL PCSK9 ANTISENSE DRUG IN HUMANS, TARGETING RESIDUAL CHOLESTEROL RISK

EP van Poelgeest, M Moerland, M Hodges, Y Tessier, AC Cohen, J Burggraaf.
Antisensemediated reduction of Proprotein Convertase Subtilis in/Kexin type 9 (PCSK9): a first-inhuman randomized, placebo-controlled trial.
British Journal of Clinical Pharmacology 2015. Dec;80(6):1350-61.

ABSTRACT

AIMS LDL-Receptor expression is inhibited by the protease proprotein convertase subtilisin/kexin type 9 (PCSK9), which is considered a pharmacological target to reduce LDL-C concentrations in hypercholesterolemic patients. We performed a first-in-human trial with SPC5001, a locked nucleic acid antisense inhibitor of PCSK9.

METHODS In this randomized, placebo-controlled trial, 24 healthy volunteers received three weekly subcutaneous administrations of SPC5001 (0.5, 1.5 or 5 mg/kg) or placebo (SPC5001:placebo ratio 6:2). End points were safety/tolerability, pharmacokinetics and efficacy of SPC5001.

RESULTS SPC5001 plasma exposure ($AUC_{0\text{-}24hr}$) increased more than dose proportionally. At 5 mg/kg, SPC5001 decreased target protein PCSK9 (day 15 to day 35: -49% vs. placebo, $P < 0.0001$), resulting in a reduction in LDL-C concentrations (maximal estimated difference at day 28 compared with placebo -0.72 mmol/L, 95% confidence interval -1.24, -0.16 mmol/L; $P < 0.01$). SPC5001 treatment (5 mg/kg) also decreased ApoB ($P = 0.04$) and increased ApoA1 ($P = 0.05$). SPC5001 administration dose-dependently induced mild to moderate injection site reactions in 44% of the subjects, and transient increases in serum creatinine of ≥20 μmol/L (15%) over baseline with signs of renal tubular toxicity in four out of six subjects at the highest dose level. One subject developed biopsy proven acute tubular necrosis.

CONCLUSIONS SPC5001 treatment dose-dependently inhibited PCSK9 and decreased LDL-C concentrations, demonstrating human proof-of-pharmacology. However, SPC5001 caused mild to moderate injection site reactions and renal tubular toxicity, and clinical development of SPC5001 was terminated. Our findings underline the need for better understanding of the molecular mechanisms behind the side effects of compounds such as SPC5001, and for sensitive and relevant renal toxicity monitoring in future oligonucleotide studies.

INTRODUCTION

Statin therapy is one of the best-proven interventions in patients at high risk for cardiovascular disease. However, target low density lipoprotein cholesterol (LDL-C) goal concentrations are not always reached. Increasing statin dose to achieve lower LDL-C concentrations may cause adverse events such as skeletal muscle aches and increases in liver enzymes and, very rarely, rhabdomyolysis [1]. Hence, novel treatments to reduce LDL-C with a different mechanism of action could be of value. Modulation of LDL-Receptor (LDL-R) expression is an attractive mechanism as LDL-C concentrations depend largely on expression and activity of the hepatic LDL-R [2].

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a protease that mediates endosomal / lysosomal degradation of the LDL-R by prolonging its retention in endosomes, resulting in reduced LDL-R recycling to the cell surface [3]. Gain-of-function mutations in PCSK9 are associated with a severe phenotype of autosomal dominant familial hypercholesterolemia [4], whereas loss-of-function mutations can result in a 30% lowering of LDL-C [5] and a 47-88% reduction in cardiovascular disease risk [6] in the absence of any other apparent phenotypic change in humans [7]. Preclinical data have shown that inhibition of PCSK9 results in reduction of serum LDL-C concentrations [8, 9], and recent clinical studies have demonstrated PCSK9-antibody mediated reductions in LDL-C concentrations and cardiovascular events [10, 11].

SPC5001 is a 14-mer oligonucleotide with locked nucleic acid (LNA) modifications. The oligonucleotide contains β-D-oxy-LNA (three locked nucleotides in both termini), and eight deoxynucleotides, arranged in the sequence 5'-TG-mCtacaaaacmCmCA-3' (where upper case letters are LNAs and mC stands for 5-methyl-LNAcytidine). Each of the internucleotide linkages is modified with phosphorothioate rather than the native phosphodiester. The LNA modified nucleotides increase the binding affinity for the target and increase nuclease resistance, thereby improving the drug-like properties [12]. SPC5001 is complementary to human PCSK9 mRNA, acts as an antisense inhibitor, and subsequently reduces intra- and extracellular PCSK9 protein levels [8]. Subcutaneously (sc) administered SPC5001 for 13 weeks in mice (maximal dose 24 mg/kg/week) and nonhuman primates (NHP) (maximal maintenance dose 20 mg/kg/week) demonstrated no rate-limiting toxicity on liver and kidney function, only minimal sc injection site reactions and no effect on coagulation parameters (Santaris Pharma A/S preclinical package, data not shown). As with other antisense oligonucleotide (AON) compounds, renal histopathology showed tubular basophilic granules suggestive of oligonucleotide drug accumulation at SPC5001 dose levels up to 20/24 mg/kg, without signs of functional nephrotoxicity. The maximum safe starting dose for clinical testing of

SPC5001 was 2.0 mg/kg/week, based on the absence of adverse effects at the highest dose tested in a 13 week NHP study (20 mg/kg). Administration of four SPC5001 doses of 6 mg/kg resulted in reductions of plasma PCSK9 protein concentrations (37%), hepatic PCSK9 mRNA levels (40%), and serum lipids (32%) in healthy NHPs. A lower dose of 1.5 mg/kg, administered once every 5 days, also induced significant reductions in LDL-C (16%), but not in plasma PCSK9. Based on these observations, three weekly SPC5001 injections of 0.5 mg/kg were considered to be an appropriate starting dose for a first-in-human (FIH) trial, on which we report here. Ascending doses of SPC5001 were administered to healthy volunteers with slightly increased fasting LDL-C concentrations (exceeding 2.59 mmol/L or 100 mg/dL), with a view to enable assessment of the human pharmacology of SPC5001 at the earliest clinical stage. Plasma PCSK9 concentrations and key lipid parameters were evaluated, together with standard safety end points and pharmacokinetic profiling.

METHODS

The study was approved by the Central Committee on Research involving Human Subjects of The Netherlands (Centrale Commissie Mensgebonden Onderzoek; CCMO, EUDRACT number of the study is 2011-000 489-36), and conducted according to the principles of the International Conference on Harmonization and Good Clinical Practice and the Helsinki Declaration. The volunteers gave written informed consent prior to screening.

STUDY DESIGN The study was randomized, ascending dose, double blind and placebo-controlled, with an SPC5001 : placebo ratio of 6 : 2 per cohort conducted at the foundation Centre for Human Drug Research, Leiden, The Netherlands. Given the exploratory character of the study, no formal power calculations were performed to assess sample size. Drug was administered subcutaneously in the abdominal region as three weekly doses of 0.5, 1.5 or 5 mg/kg on study days 1, 8 and 15 (150 mg/ml SPC5001 dissolved in water for injection; injection volumes ≤3 ml administered in a single injection and volumes >3 ml in two injections). The starting dose of 0.5 mg/kg/week was 4-fold lower than the maximum recommended starting dose of 2.0 mg/kg/week, based on a NOAEL of 20 mg/kg per dose in NHPs and a human equivalent dose (HED) of 20 mg/kg per dose, with an applied safety factor of 10. After the first and last SPC5001 or placebo (0.9% saline) administration, the subjects were confined to the clinical research unit for 24hrs. After the second administration the subjects were monitored for 4hrs. The last follow-up visit was conducted on study day 78.

STUDY PARTICIPANTS Thirty-two healthy volunteers, aged 18 to 65 years with a fasting LDL-C of ≥2.59 mmol/L (≥ 100 mg/dL) and triglycerides ≤ 4.5 mmol/L (≤ 398 mg/dL), a body mass index (BMI) of 18-33 kg/m^2, without ultrasonographic signs of liver steatosis and not using concomitant medication, were planned to be enrolled in four subsequent cohorts.

SAFETY AND TOLERABILITY Safety monitoring was performed by adverse event monitoring, physical examination, assessment of ECG and vital signs, and laboratory evaluations (routine hematology, chemistry including C-reactive protein and gamma globulins, coagulation, complement factors, cytokines, and semi quantitative dipstick urinalysis). In case of clinically significant findings in dipstick analysis, a microscopic investigation of the urine was performed. Hepatic ultrasonography (Siemens P50) was performed at screening and the last follow-up visit for exclusion of hepatic steatosis. Dosing for each subsequent cohort commenced only after satisfactory review of 16 day safety data from the preceding cohort. Post hoc analysis of exploratory kidney injury biomarkers was performed for urinary β2-microglobulin (Immulite 2000, a solid-phase two-side chemiluminescent immunometric assay), αGST (Argutus Medical Alpha GST EIA enzyme immunoassay), NAG (Diazyme europe GmbH) and KIM1 (Quantikine human TIM-1/KIM-1/HAVCR immunoassay). Samples for these biomarkers were collected on study days 1, 8 and 15 and stored at -80°C until analysis. Additional blood and urine samples were collected upon renal safety concerns that appeared during study conduct.

PHARMACODYNAMICS Throughout the study, pharmacodynamic effects of SPC5001 were assessed in fasting blood samples by measurement of PCSK9, TC, HDL-C, TG, ApoA1, ApoB and VLDL-C. Total (LDL-bound and -unbound) PCSK9 was measured using the circulex human PCSK9 ELISA kit. The sensitivity was 0.154 ng/L and the coefficient of variation was -3%. LDL-C was calculated according to the Friedewald formula: LDL-C = TC – HDL-C – (0.456*TG). VLDL-C was calculated as TC – HDL-C – LDL-C.

PHARMACOKINETICS For the quantification of SPC5001, plasma samples (collected frequently on dosing days 1 and 15, pre-dose on day 8 and during follow-up visits) were analyzed by a validated hybridization-dependent ELISA method (Santaris Pharma A/S, Technical Report), with a lower limit of quantitation (LOQ) of 0.4 ng/mL. The overall coefficient of variation was ~9%. In addition, urine samples collected on dosing days 1 and 15 (pre-dose and 0-4, 4-8 and 8-24 h post-dose) were analyzed by a comparable qualified method. SPC5001

plasma concentrations were subjected to non-compartmental pharmacokinetic evaluation in order to determine the maximum observed plasma concentration (C_{max}), the time to maximum plasma concentration (T_{max}) and the area under the plasma concentration-time curve from dosing to 24 h after dosing ($AUC_{0\text{-}24hr}$) using WinNonLin (version 5.3, Pharsight Corporation, USA). Urine was collected prior to dose and during 24hrs after the first and the third doses (on days 1 and 15, respectively) from all subjects (except one subject dosed at 1.5 mg/kg per dose on day 15). The concentration of SPC5001 in the urine was quantified by the hybridization-dependent ELISA method and the amount of the compound in relation to the dose was calculated.

STATISTICAL METHODS AND ANALYSIS Descriptive statistics were used to summarize demographic and baseline characteristics. Statistical analysis was performed for all pharmacodynamic parameters. To correct for the expected log-normal distribution, PCSK9, HDL-C, TG and VLDL-C were log-transformed prior to analysis. All repeatedly measured pharmacodynamic parameters were analyzed with a mixed model of variance with fixed factors treatment, time and treatment by time and random factor subject, and with the baseline measurement on day 1 as covariate. Contrasts between each SPC5001 dose level and placebo were calculated for a study period up to and including day 49 (the time span in which SPC5001 exerted the intended effects, at least at the highest dose level tested), unless otherwise indicated. All analyses were performed using SAS for Windows Version 9.1.3 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

PARTICIPANT CHARACTERISTICS Twenty-four subjects were enrolled in the study. Demographics are summarized in Table 1. One subject in the 1.5 mg/kg dose group was withdrawn after administration of two SPC5001 doses due to non-compliance with the study lifestyle rules. Therefore, 23 subjects completed the study, receiving three doses of SPC5001 or placebo.

SAFETY SUMMARY One participant dosed at the highest dose (5 mg/kg per dose) experienced acute tubular necrosis [11]. Therefore, follow-up was intensified for this subject and all other participants still under follow-up. Upon review of elevated serum creatinine values together with urine sediment analyses, the Safety Review Committee decided to stop further dose escalation. For this reason, only 24 subjects were enrolled in the study and not the anticipated 32. In addition, a more comprehensive set of kidney injury biomarkers was retrospectively analyzed. The most frequent occurring adverse events were injection site reactions (ISRs), observed in 44% of the SPC5001-treated subjects and in none of the placebo treated subjects.

RENAL EFFECTS SPC5001 dose-dependently increased serum creatinine. Whereas in the 0.5 and 1.5 mg/kg per dose groups no clinically relevant effects on serum creatinine levels were observed, SPC5001 treatment at 5 mg/kg per dose induced a transient increase in serum creatinine (Figure 1, Table 2, P = 0.02), which was observed in four out of six subjects. Average serum creatinine concentrations in that group started to increase after the last SPC5001 administration and peaked approximately 10 days after the final dose (from 84 ± 12 to 106 ± 15 μmol/L; reference ranges are 64-104 μmol/L for males and 49-90 μmol/L for females). Subsequently, serum creatinine gradually declined to baseline levels. The rise in serum creatinine coincided with the appearance of urinary granular casts. In addition, one of the subjects developed acute tubular necrosis 5 days after the last SPC5001 administration, which has been described in detail in a case report [13]. Upon appearance of these renal effects, additional blood and urinary samples were collected in all volunteers who were still under follow-up. Both the scheduled samples (collected pre-dose, and in weeks 1, 2 and 11) and the additional samples were analyzed for serum creatinine, urinary β2-microglobulin, α-glutathione S-transferase (αGST), kidney injury molecule-1 (KIM1) and N-acetyl-β-D-glucosaminidase (NAG). Increases in serum creatinine (9%), urinary β2-microglobulin (200%), and urinary KIM1 (55%) were observed at the highest SPC5001 dose level tested (5 mg/kg/week for 3 weeks) (Table 2), with serum creatinine and urinary KIM1 reaching peak concentrations at the fourth week (Figures 1 and 2). However, the collection of these data was not balanced between the treatment groups. In the 5 mg/kg dosing group, the elevations in serum creatinine, β2-microglobulin and αGST reached statistical significance (Table 2). It should be noted that the increases in β2-microglobulin were isolated, transient and highly variable in timing between subjects and did not correlate with the observed changes in other tubular markers. No SPC5001-related changes were observed in NAG (Table 2).

INJECTION SITE REACTIONS Injection site reactions (ISRs) developed dose-dependently in 8/18 (44%) of the SPC5001-treated subjects (0/6, 3/6 and 5/6 subjects in the 0.5, 1.5 and 5 mg/kg dosing groups, respectively). These skin reactions presented hours to days after the sc injections as painless erythema at the site of the injection (approximately 5 cm by 5 cm) with or without transient

pruritus and/or swelling. Most skin lesions became clearly visible 1 week after the last SPC5001 administration. Subjects either had skin lesions at all three locations of the abdomen where SPC5001 had been administered, or they had no skin lesions at all. The ISRs did not worsen with each subsequent injection, and all skin lesions within a subject were of similar severity. The ISRs were of mild to moderate severity and caused significant discomfort to a subset of the volunteers. In one female, a maculopapular rash developed 1 week after the final dose. The patient was referred to a dermatologist who treated the patient with topical steroids resulting in resolution of the rash. In general, the ISRs persisted for several days to weeks and then diminished in intensity. However, in one female, subcutaneous skin atrophy developed, which was present at the final visit 2.5 months after dosing. Also, in six out of eight SPC5001-treated subjects (three subjects from 1.5 mg/kg group and three subjects from 5 mg/kg group) skin hyperpigmentation was present at the last follow-up visit at 2.5 months.

OTHER SAFETY ASSESSMENTS SPC5001 treatment did not result in clinically relevant changes in vital signs, ECG parameters, or coagulation, or in (trends to) increases in complement factors, cytokines or CRP, neither in subjects free of ISRs nor in subjects developing ISRs. Also, no clinically relevant changes in liver biochemistry parameters were observed, and none of the treated subjects had ultrasonographic signs of steatosis of the liver parenchyma at the last follow-up visit. Adverse events that were observed more frequently in SPC5001-treated subjects than in placebo treated subjects were mild headache (61 vs. 33%) and tiredness (56 vs. 17%), occurring not dose dependently throughout the complete study period with a higher incidence within the first 24 h after SPC5001/placebo administration, and generally spontaneously resolving within hours to days.

PHARMACOKINETICS Maximal plasma concentrations were reached at 1.7 ± 0.5, 1.2 ± 0.4, and 2.5 ± 2.7 hrs post-dose for 0.5, 1.5 and 5 mg/kg SPC5001, respectively (mean ± SD). The maximal plasma concentrations increased dose-proportionally (281 ± 43, 757 ± 32, and 2424 ± 692 ng ml-1 for 0.5, 1.5 and 5 mg kg-1, respectively), while $AUC_{0\text{-}24hr}$ increased more than dose-proportionally (1.78 ± 0.13, 5.01 ± 0.46 and 23.0 ± 3.8 µg/mL/h for 0.5, 1.5 and 5 mg/kg, respectively). The rate constants of the terminal phases describing the decline in SPC5001 plasma concentration were not formally calculated, but the half-life of the final phase was estimated to be 7 days. SPC5001 excreted in urine was determined in samples collected during 0-24 h after dosing on days 1 and 15. The total amount of SPC5001 in urine increased more than dose-proportionally (Figure 3).

PHARMACODYNAMICS SPC5001 treatment resulted in a decrease in PCSK9 plasma concentration, reaching a level of significance when compared with placebo at the highest dose levels tested (Figure 4, Table 3). The maximal decrease in PCSK9 concentration was reached 1 week after the last SPC5001 administration (Figure 4; from 302 ± 80 ng/mL at baseline to 156 ± 85 ng/mL on day 21, at 5 mg/kg SPC5001), remaining decreased until at least the last measurement point on day 35 (Figure 4). SPC5001 also induced a dose-dependent decrease in LDL-C concentrations, with a maximal effect reached 2 weeks after the last SPC5001 administration (Figure 5, from 3.8 ± 0.8 mmol/L at baseline to 2.9 ± 1.1 mmol/L on day 29, for 5 mg/kg SPC5001). Although the observed effect was not significant for the full time profile (Table 3), the contrast between 5 mg/kg SPC5001 and placebo was statistically significant at day 28 (estimated difference -0.72 mmol/L, 95% confidence interval -1.24, -0.16 mmol/L; $P < 0.01$). LDL-C concentrations returned to baseline 9 weeks after administration of the last SPC5001 dose. Furthermore, 5 mg/kg SPC5001 decreased apolipoprotein B (ApoB) (Table 3, $P = 0.05$ vs. placebo), with a maximal average decrease from baseline of approximately 15% (0.17 g/L) observed 1 week after the last administration (data not shown), and increased apolipoprotein A1 (ApoA1) (Table 3, $P = 0.04$ vs. placebo), with a mean maximal average increase over baseline of 8% (0.13 g/L) observed at study day 14 (data not shown). High-density lipoprotein cholesterol (HDL-C), triglycerides (TG) and very low density lipoprotein cholesterol (VLDL-C) concentrations were unaffected by SPC5001 treatment (Table 3).

DISCUSSION

This study explored the pharmacokinetics and LDL-C lowering effects of SPC5001, an LNA-based PCSK9-targeted antisense oligonucleotide, in healthy volunteers with moderately elevated LDL-C concentrations. Proof-of-pharmacology for SPC5001 was achieved. SPC5001 administration resulted in a reduction of plasma PCSK9 and a lowering of LDL-C and ApoB. However, the top dose (5 mg/kg) not only resulted in a maximal decrease in PCSK9 concentration of approximately 50% and a reduction in LDL-C of maximally 25% compared with baseline, but also in renal tubular effects. It is unknown whether the more than dose-proportional increase in SPC5001 plasma exposure ($AUC_{0\text{-}24hr}$) and urinary excretion, possibly representing a limitation in SPC5001's hepatic uptake/accumulation, relates to the observed nephrotoxicity.

Healthy cynomolgus monkeys dosed at similar or higher levels displayed the intended pharmacology (i.e. reduction in LDL-C) but no indications of renal

toxicity (Santaris Pharma A/S, unpublished observations). In these animals, SPC5001 dosing (loading dose of 20 mg kg1, followed by four weekly doses of 5 mg/kg) reduced hepatic PCSK9 messenger RNA and plasma PCSK9 protein concentrations by up to 85%, and circulating LDL-C by up to 50% without affecting routine renal markers (urea and creatinine) [9]. Also in a 13-week toxicity study with a loading phase of up to 20 mg/kg per dose every 5th day for 16 days followed by a maintenance phase of 20 mg/kg/week, neither clinical chemistry nor histopathology pointed to a tubular risk (Santaris Pharma A/S, unpublished GLP study report). However, interspecies differences in drug sensitivity are not unusual, hence the safety margins conventionally applied from animals to humans. PCSK9 inhibition per se is unlikely to be the cause for the observed renal tubular toxicity observed in our study. Other PCSK9-inhibiting modalities tested in clinical studies have not resulted in renal signals. Inhibition of PCSK9-synthesis by a single dose of silencing RNA was demonstrated to be a potentially safe and effective strategy, with a mean 70% reduction in circulating PCSK9 plasma protein (P < 0·0001) and a mean 40% reduction in LDL cholesterol from baseline relative to placebo (P < 0·0001) [14]. Furthermore, no renal toxicity has been reported for plasma PCSK9-directed antibodies, resulting in decreases in plasma PCSK9 concentrations up to 100% and reductions in LDL-C between 60 and 80% in phase 1 trials [15, 16]. Finally, there are no reports to our knowledge of functional renal changes in people with loss-of-function PCSK9 mutations [17]. The renal effects of SPC5001 included a transient increase in serum creatinine, with first onset after the last SPC5001 administration and peaking approximately 10 days after the final dose. This coincided with the appearance of urinary granular casts, and elevations of urinary kidney damage markers. One subject in the highest dose group developed acute tubular necrosis (ATN), which resolved spontaneously within 8 weeks. The observation of ATN is uncommon for unmodified oligodeoxynucleotides, 2'-MOE modified and LNA modified oligonucleotides, which have all been successfully administered to humans without causing clinically meaningful renal functional changes [18-20]. The target-unrelated toxicity of individual oligonucleotides is diverse and probably driven by a range of factors including backbone and nucleoside chemistry, sequence and length. The mechanism behind renal toxicity associated with some oligonucleotides is currently unknown. It may relate to accumulation-related degenerative effects on the proximal tubule although in animal models molecules that accumulate most are not necessarily the most toxic. For instance, miravirsen accumulated to a 8-fold higher degree than SPC5001 in NHP kidney cortex and showed no renal toxicity in humans (Santaris Pharma A/S, Technical Reports and [19]). Other factors such as individual susceptibility of the patient may be involved as well. It is important to note that adverse renal effects may also occur in AONs with chemistries other than LNA. There are reports on second generation AON induced proteinuria in patients with Duchenne's muscular dystrophy [21] and ATN in a metastatic cancer patient [22]. It has also been previously stated in the literature that shorter oligonucleotides have lower plasma protein binding capability and, therefore, a larger proportion of the dose may pass the glomerulus and accumulate in the proximal tubular cells [23]. However there is no relation, to our knowledge, between the rate of filtration and the level of tubular accumulation. Moreover, SPC5001 is a relatively short molecule (14-mer) but shows a high binding (of >95%) to human serum albumin (Santaris Pharma A/S, internal data). In the current trial, only 1-3% of the dose was excreted in urine during the first 24 h after administration (approximately corresponding to the unbound fraction), which is low compared with a 24 h urinary dose recovery of 8-10% for miravirsen, a 15-mer oligonucleotide that did not induce tubulotoxicity in early clinical trials [24, 25]. Because oligonucleotides accumulate in the proximal tubules to varying degrees [26], renal monitoring is performed for investigational compounds of this drug class. Not all clinical adverse events can be predicted preclinically and it is therefore suggested that extensive renal safety monitoring for AONs should not be limited to routine measures such as serum creatinine and urinalysis, but should also include regular urine microscopy. In addition, measurement of the urinary excretion of specific tubular damage markers such as KIM1, β2-microglobulin, αGST, and possibly NAG may also be informative. Thorough assessment of renal damage markers may allow early detection of impending renal damage and possibly provide mechanistic insight [27], which is crucial for the understanding of why some, but not all, oligonucleotides have been demonstrated to induce unintended renal effects. These proposed urinary biomarkers are not fully clinically validated, and while their use is encouraged, they may not yet be ready to support real-time decision-making. Since the observed renal effects of SPC5001 in this clinical study, the stringency of renal safety screening of LNA oligonucleotides has increased. A recent rat-based standard study including both routine and advanced biomarkers reproduced the tubulotoxicity of SPC5001 [28]. Further mechanistic studies are in progress to understand the factors causing this type of toxicity.

SPC5001 treatment dose-dependently induced mild to moderate ISRs. The occurrence of ISR suggests that SPC5001, like other charged phosphorothioate oligonucleotides [29], has the potential to induce local (subcutaneous) inflammatory changes. In the present study, there was no evidence for systemic inflammation as indicated by the absence of elevations in C-reactive protein and gamma globulins,

circulating cytokines or complement activation (data not shown). It has been reported that ISRs do occur in rodents and to a lesser extent in primates but also that animal models poorly predict this risk in humans [30]. Subcutaneous administration of SPC5001 in preclinical models resulted in only minimal injection site effects, so the toxicology studies did not predict the magnitude of ISRs upon SPC5001 treatment in humans. However, the occurrence of AON-induced ISRs in humans has been reported before. During the clinical development of mipomersen (2'-MOE phosphorothioate AON), 84% of all mipomersen-treated patients displayed ISRs compared with 33% of all placebo-treated patients [31]. Furthermore, PRO051 (a fully modified 2'-O-methyl phosphorothioate AON) administered to patients with Duchenne's muscular dystrophy resulted in erythema and inflammation at the injection site in 9 out of 12 treated patients [21]. This shows that phosphorothioate AONs have the potential to induce injection site reactions. It is currently unknown how sequence and structure of AON compounds relates to the nature of skin lesions and dose, concentration and volume are factors that may modulate these effects. However, a wide range of ISR-inducing potencies has been observed across development compounds (Santaris Pharma A/S, unpublished observations). The increased potency and duration of action of next-generation antisense oligonucleotides (and related less frequent dosing regimens and smaller injection volumes) may reduce the risk of ISR development [32-34]. Data need to be generated more systematically and for that purpose it is essential that injection site reactions are documented in more detail, preferably following a standardized (AON-specific) scoring system. Furthermore, the underlying pathophysiology of ISRs remains poorly understood.

In conclusion, SPC5001 administered to healthy volunteers with mildly elevated LDL-C resulted in dose dependent reductions in LDL-C and PCSK9. However, subcutaneous injections of SPC5001 were associated with ISRs and transient renal tubular toxicity at 5 mg/kg. It is now recognized that within the LNA chemical class, these toxicities vary greatly across compound sequences and further screens have been or are being phased in to reduce those risks at the discovery stage [28]. The ultimate goal is to obtain insights into the molecular mechanisms behind the side effects of compounds such as SPC5001, with a view to improve the quality of compound libraries. In the same spirit, sensitive and relevant monitoring strategies for renal toxicity should be implemented for future oligonucleotide therapies.

Table 1. Subject Baseline Demographics, average (SD)

		SPC5001 (N=18)		Placebo (N=6)
	0.5 mg/kg (N=6)	1.5 mg/kg (N=6)	5 mg/kg (N=6)	
Gender (M:F)	5:1	4:2	2:4	1:5
Age (yrs)	36 (14)	52 (14)	53 (15)	50 (15)
Weight (kg)	82 (11)	73 (10)	74 (16)	70 (13)
BMI (kg/m^2)	25 (2)	24 (3)	25 (4)	25 (3)
LDL-C (mmol/L)	3.02 (0.56)	4.00 (0.92)	4.26 (0.73)	3.82 (0.44)
Creatinine (µmol/L)	78 (14)	84 (11)	80 (14)	70 (8)

Table 2. Serum creatinine and urinary kidney injury markers.
Estimated differences between active treatment and placebo, calculated for scheduled samples over the complete study period of 78 days, expressed as % change, presented with 95% confidence interval and corresponding P value.

	0.5 mg/kg SPC5001 vs Placebo N=6	1.5 mg/kg SPC5001 vs Placebo N=6	5 mg/kg SPC5001 vs Placebo N=6	Placebo average (SD) N=6
Serum Creatinine (µmol/L)	7.0% (0.5-14%) p=0.04 N=109	7.5% (0.6-14.8%) p=0.03 N=105	8.6% (1.6-16.2%) p=0.02 N=136	70 (8) N=115
Urinary Beta 2 Microglobulin (µg/L)	41.5%(-35.4-210.2%) p=0.4 N=25	114.4%(-0.3-361%) p=0.05 N=34	197.2%(32.4-566.8%) p=0.01 N=68	46 (35) N=38
Urinary KIM1 (ng/mL)	-20.6% (-59.2-54.4%) p=0.5 N=24	25.5%(-36.7-149%) p=0.5 N=28	55.3% (-21.5-207.1%) p=0.2 N=57	0.85 (0.66) N=35
Urinary Alpha-GST* (µg/L)	77.8%(-0.19-0.70%) p=0.2 N=21	29.0% (-0.6-0.16%) p=0.6 N=21	280.6% (-0.55-0.22%) p=0.004 N=20	9 (6) N=20
Urinary NAG (IU/L)	16.1% (-14.8-2.1%) p=0.5 N=23	52.4% (-7.2-9.8%) p=0.09 N=26	8.6% (-6.1-11.1%) p=0.7 N=50	5.92 (3.82) N=28

n: number of samples analyzed, N: number of subjects
*Urinary *α-GST* not measured in additional samples for technical reasons.

Table 3. Effects of SPC5001 treatment on lipid parameters.
Estimated differences between active treatment and placebo, calculated over a period from baseline up to and including day 49, expressed as absolute change with corresponding unit or as percentage for log transformed parameters (PCSK9, HDL-C, VLDL-C and triglycerides), presented with 95% confidence interval and corresponding P value. Estimated difference for PCSK9 was calculated from baseline up to and including day 35.

	0.5 mg/kg SPC5001 Vs Placebo	1.5 mg/kg SPC5001 vs Placebo	5 mg/kg SPC5001 vs Placebo
PCSK9 (%)	-2.5 (-20.7-20.0) p=0.8	-15.0 (-31.0-4.7) p=0.1	-49.0 (-58.5--37.2) p<0.0001
ApoA1 (g/L)	0.04 (-0.07-0.15) p=0.5	0.01 (-0.09-0.12) p=0.8	0.11 (0.01-0.21) p=0.04
ApoB (g/L)	-0.02 (-0.11-0.07) p=0.6	0.01 (-0.08-0.09) p=0.9	-0.09 (-0.18-0.00) p=0.05
Total cholesterol (mmol/L)	0.24 (-0.20-0.69) p=0.3	-0.22 (-0.60-0.17) p=0.3	-0.19 (-0.57-0.20) p=0.3
HDL-C (%)	-6.9 (-15.0-1.9) p=0.1	0.7 (-7.5-9.6) p=0.9	2.8 (-5.5-11.8) p=0.5
LDL-C (mmol/L)*	0.06 (-0.37-0.50) p=0.8	-0.18 (-0.57-0.21) p=0.4	-0.36 (-0.75-0.03) p=0.07
VLDL-C (%) #	34.7 (-2.3-85.6) p=0.06	2.9 (-25.8-42.5) p=0.9	22.8 (-11.7-70.8) p=0.2
Triglycerides (%)	34.5 (-2.2-84.6) p=0.07	3.2 (-25.3-42.5) p=0.8	23.0 (-11.3-70.6) p=0.2

* calculated with Friedewald formula: LDL-C = (TC)-(HDL-C)-(0.456*Triglycerides).
# calculated as: (total cholesterol)-(HDL-cholesterol)-(indirect LDL-cholesterol).

Figure 1. Average serum creatinine concentrations (µmol/L), with SD bars.
SPC5001 administration was on study days 1, 8 and 15. Averages include both scheduled and unscheduled samples, grouped by week.

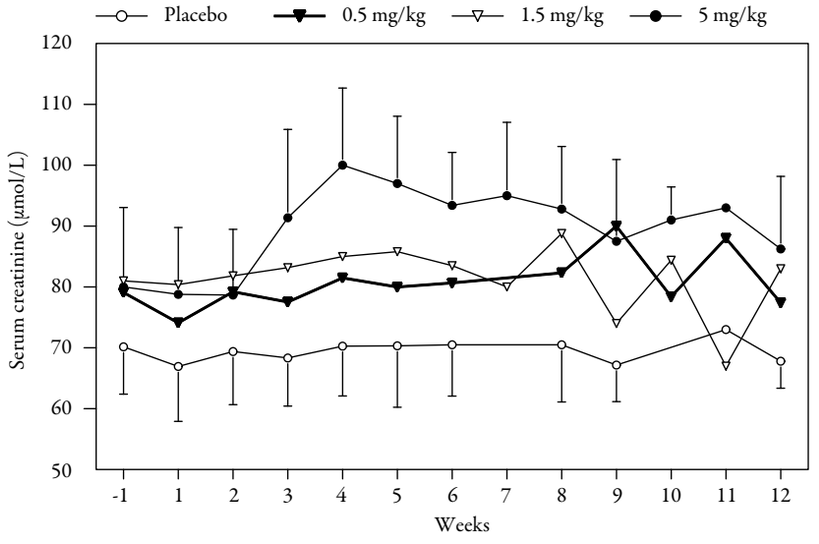


Figure 2. Average urinary kidney injury molecule-1 concentrations (ng/mL), with SD bars.
SPC5001/placebo administration was on study days 1, 8 and 15. Averages include both scheduled and unscheduled samples, grouped by week.

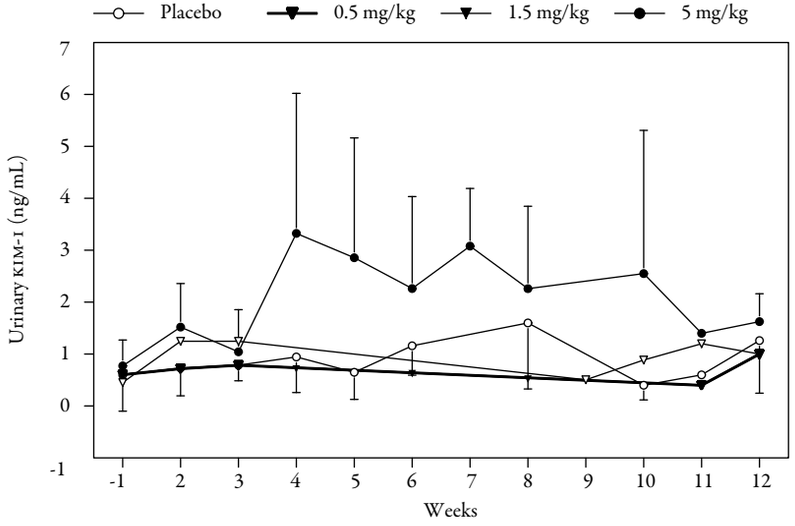


Figure 3. Average SPC5001 urinary excretion (mg) over 24 h after dosing, with SD bars.
n = 6 subjects/dose level, except for 1.5 mg/kg (n = 5).

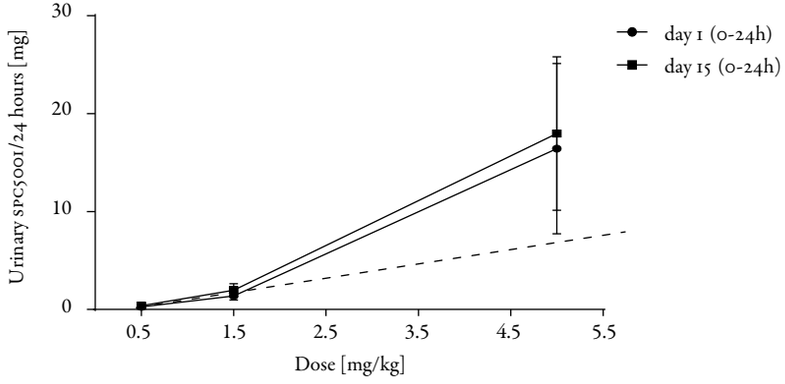


Figure 4. Average estimated PCSK9 concentrations (ng/mL), expressed as % change from baseline with 95% confidence interval bars.

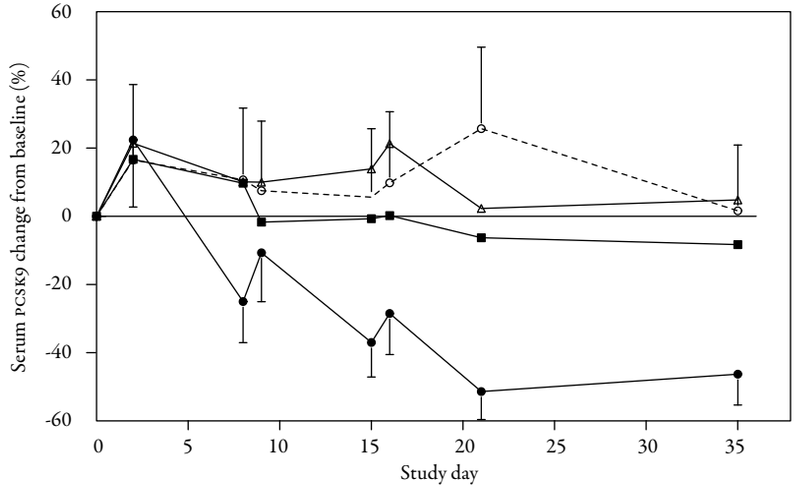
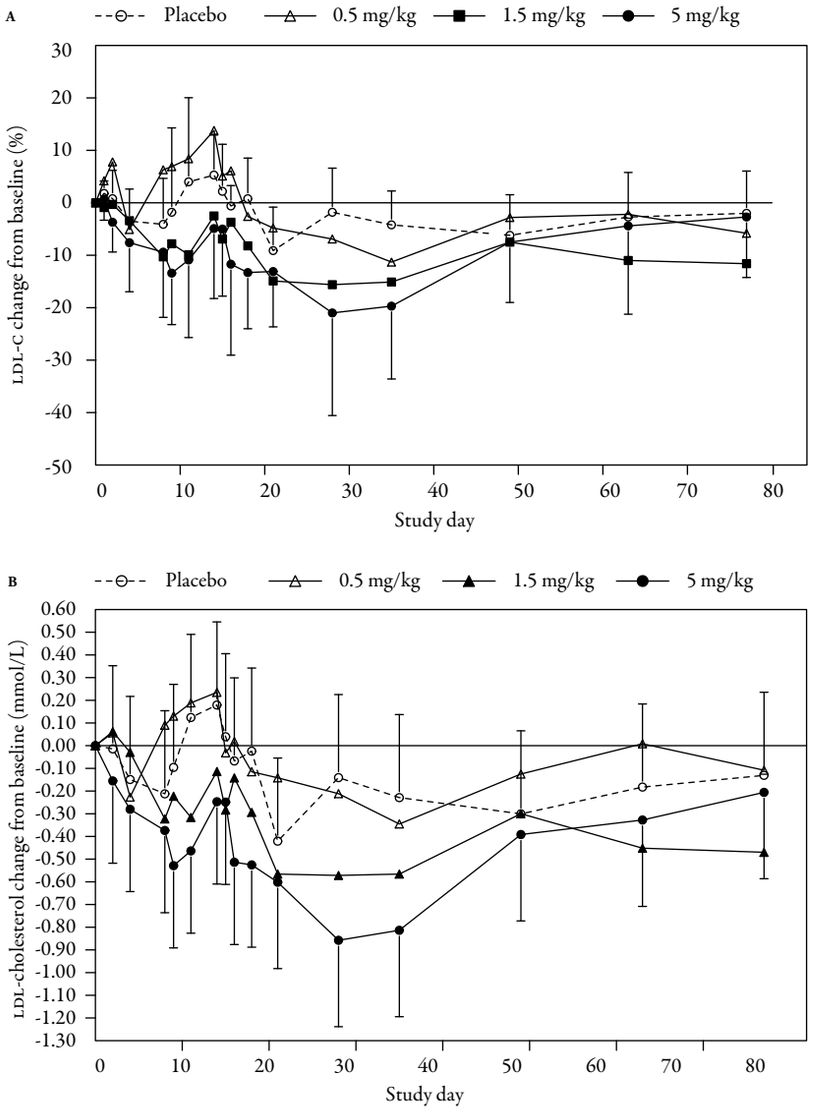


Figure 5. Average estimated LDL-cholesterol concentrations in mmol/L (A) and in percentage (B), expressed as change from baseline with 95% confidence interval bars.



REFERENCES

1 Silva MA, Swanson AC, Gandhi PJ, Tataronis GR. Statin-related adverse events: a meta-analysis. Clin Ther 2006; 28: 26-35.

2 Goldstein JL, Brown MS. The LDL receptor. Arterioscler Thromb Vasc Biol 2009; 29: 431-8.

3 Nassoury N, Blasiole DA, Tebon OA, Benjannet S, Hamelin J, Poupon V, McPherson PS, Attie AD, Prat A, Seidah NG. The cellular trafficking of the secretory proprotein convertase PCSK9 and its dependence on the LDL-R. Traffic 2007; 8: 718-32.

4 Abifadel M, Varret M, rabes JP, Allard D, Ouguerram K, Devillers M, Cruaud C, Benjannet S, Wickham L, Erlich D, Derre A, Villeger L, Farnier M, Beucler I, Bruckert E, Chambaz J, Chanu B, Lecerf JM, Luc G, Moulin P, Weissenbach J, Prat A, Krempf M, Junien C, Seidah NG, Boileau C. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat Genet 2003; 34: 154-6.

5. Tibolla G, Norata GD, Artali R, Meneghetti F, Catapano AL. Proprotein convertase subtilisin/kexin type 9 (PCSK9): from structure-function relation to therapeutic inhibition. Nutr Metab Cardiovasc Dis 2011; 21: 835-43.

6 Cohen JC, Boerwinkle E, Mosley TH Jr, Hobbs HH. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. N Engl J Med 2006; 354: 1264-72.

7 Cohen J, Pertsemlidis A, Kotowski IK, Graham R, Garcia CK, Hobbs HH. Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. Nat Genet 2005; 37: 161-5.

8 Gupta N, Fisker N, Asselin MC, Lindholm M, Rosenbohm C, Orum H, Elmen J, Seidah NG, Straarup EM. A locked nucleic acid antisense oligonucleotide (LNA) silences PCSK9 and enhances LDL-R expression *in vitro* and *in vivo*. PLoS One 2010; 5: e10682.

9 Lindholm MW, Elmen J, Fisker N, Hansen HF, Persson R, Moller MR, Rosenbohm C, Orum H, Straarup EM, Koch T. PCSK9 LNA antisense oligonucleotides induce sustained reduction of LDL cholesterol in nonhuman primates. Mol Ther 2012; 20: 376-81.

10 Navarese EP, Kolodziejczak M, Schulze V, Gurbel PA, Tantry U, Lin Y, Brockmeyer M, Kandzari DE, Kubica JM, D'Agostino RB Sr, Kubica J, Volpe M, Agewall S, Kereiakes DJ, Kelm M. Effects of proprotein convertase subtilisin/kexin type 9 antibodies in adults with hypercholesterolemia: a systematic review and meta-analysis. Ann Intern Med 2015;163: 40-51.

11 Robinson JG, Farnier M, Krempf M, Bergeron J, Luc G, Averna M, Stroes ES, Langslet G, raal FJ, El SM, Koren MJ, Lepor NE, Lorenzato C, Pordy R, Chaudhari U, Kastelein JJ. Efficacy and safety of alirocumab in reducing lipids and cardiovascular events. N Engl J Med 2015; 372: 1489-99.

12 Veedu RN, Wengel J. Locked nucleic acid as a novel class of therapeutic agents. RNA Biol 2009; 6: 321-3.

13 van Poelgeest EP, Swart RM, Betjes MG, Moerland M, Weening JJ, Tessier Y, Hodges MR, Levin AA, Burggraaf J. Acute kidney injury during therapy with an antisense oligonucleotide directed against PCSK9. Am J Kidney Dis 2013; 62: 796-800.

14 Fitzgerald K, Frank-Kamenetsky M, Shulga-Morskaya S, Liebow A, Bettencourt BR, Sutherland JE, Hutabarat RM, Clausen VA, Karsten V, Cehelsky J, Nochur SV, Kotelianski V, Horton J, Mant T, Chiesa J, Ritter J, Munisamy M, Vaishnaw AK, Gollob JA, Simon A. Effect of an RNA interference drug on the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) and the concentration of serum LDL cholesterol in healthy volunteers: a randomised, single-blind, placebo-controlled, phase 1 trial. Lancet 2014; 383: 60-8.

15 Dias CS, Shaywitz AJ, Wasserman SM, Smith BP, Gao B, Stolman DS, Crispino CP, SmiRNakis KV, Emery MG, Colbert A, Gibbs JP, Retter MW, Cooke BP, Uy ST, Matson M, Stein EA. Effects of AMG 145 on low-density lipoprotein cholesterol levels: results from two randomized, double-blind, placebo controlled, ascending-dose phase 1 studies in healthy volunteers and hypercholesterolemic subjects on statins. J Am Coll Cardiol 2012; 60: 1888-98.

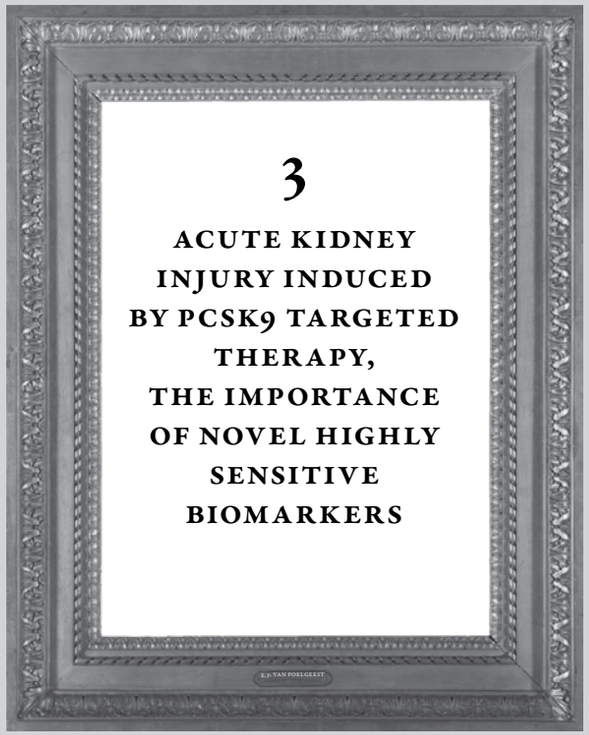
16 Stein EA, Mellis S, Yancopoulos GD, Stahl N, Logan D, Smith WB, Lisbon E, Gutierrez M, Webb C, Wu R, Du Y, Kranz T, Gasparino E, Swergold GD. Effect of a monoclonal antibody to PCSK9 on LDL cholesterol. N Engl J Med 2012; 366: 1108-18.

17 Zhao Z, Tuakli-Wosornu Y, Lagace TA, Kinch L, Grishin NV, Horton JD, Cohen JC, Hobbs HH. Molecular characterization of loss-of-function mutations in PCSK9 and identification of a compound heterozygote. Am J Hum Genet 2006; 79:514-23.

18 Crooke S. Antisense Drug Technology: Principles, Strategies, and Applications, 2nd edn. Boca raton Florida: CRC Press, 2007.

19 Janssen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K, van der Meer AJ, Patick AK, Chen A, Zhou Y, Persson R, King BD, Kauppinen S, Levin AA, Hodges MR. Treatment of HCV infection by targeting microRNA. N Engl J Med 2013; 368: 1685-1694.

20 Raal FJ, Santos RD, Blom DJ, Marais AD, Charng MJ, Cromwell WC, Lachmann RH, Gaudet D, Tan JL, Chasan-Taber S, Tribble DL, Flaim JD, Crooke ST. Mipomersen, an apolipoprotein B synthesis inhibitor, for lowering of LDL cholesterol concentrations in patients with homozygous familial hypercholesterolaemia: a randomised, doubleblind, placebo-controlled trial. Lancet 2010; 375: 998-1006.
21 Goemans NM, Tulinius M, van den Akker JT, Burm BE, Ekhart PF, Heuvelmans N, Holling T, Janson AA, Platenburg GJ, Sipkens JA, Sitsen JM, Aartsma-Rus A, van Ommen GJ, Buyse G, Darin N, Verschuuren JJ, Campion GV, de KIMpe SJ, van Deutekom JC. Systemic administration of PRO051 in Duchenne's muscular dystrophy. N Engl J Med 2011; 364: 1513-22.
22 Herrington WG, Talbot DC, Lahn MM, Brandt JT, Callies S, Nagle R, Winearls CG, Roberts IS. Association of long-term administration of the survivin mRNA-targeted antisense oligonucleotide LY2181308 with reversible kidney injury in a patient with metastatic melanoma. Am J Kidney Dis 2011; 57: 300-3.
23 Laxton C, Brady K, Moschos S, Turnpenny P, rawal J, Pryde DC, Sidders B, Corbau R, Pickford C, Murray EJ. Selection, optimization, and pharmacokinetic properties of a novel, potent antiviral locked nucleic acid-based antisense oligomer targeting hepatitis C virus interNAl ribosome entry site. Antimicrob Agents Chemother 2011; 55: 3105-14.
24 Hildebrandt-Eriksen ES, Aarup V, Persson R, Hansen HF, Munk ME, Orum H. A locked nucleic acid oligonucleotide targeting microRNA 122 is well-tolerated in cynomolgus monkeys. Nucleic Acid Ther 2012; 22: 152-61.
25 van der Ree MH, van der Meer AJ, de Bruijne J, Maan R, van Vliet A, Welzel tm, Zeuzem S, Lawitz EJ, Rodriguez-Torres M, Kupcova V, Wiercinska-Drapalo A, Hodges MR, Janssen HL, Reesink HW. Long-term safety and efficacy of microRNA targeted therapy in chronic hepatitis C patients. Antiviral Res 2014; 111: 53-9.
26 Bennett CF, Swayze EE. RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. Annu Rev Pharmacol Toxicol 2010; 50: 259-93.
27 Waring WS, Moonie A. Earlier recognition of nephrotoxicity using novel biomarkers of acute kidney injury. Clin Toxicol (Phila) 2011; 49: 720-8.
28 Lindholm MW. LNA oligonucleotide *in vivo* renal screening v2.0 (Poster), DIA/FDA meeting on oligonucleotide therapeutics, Washington, September 2013.
29 Hair P, Cameron F, McKeage K. Mipomersen sodium: first global approval. Drugs 2013; 73: 487-93.
30 Engelhardt JA. Predictivity of animal studies for human injection site reactions with parenteral drug products. Exp Toxicol Pathol 2008; 60: 323-7.
31 FDA Briefing Document NDA 203568 Mipomersen Sodium Injection 200 mg/mL (2012).
32 Frazier KS. Antisense oligonucleotide therapies: the promise and the challenges from a toxicologic pathologist's perspective. Toxicol Pathol 2015; 43: 78-89.
33 Nair JK, Willoughby JL, Chan A, Charisse K, Alam MR, Wang Q, Hoekstra M, Kandasamy P, Kel'in AV, Milstein S, Taneja N, O'Shea J, Shaikh S, Zhang L, van der Sluis RJ, Jung ME, akinc A, Hutabarat R, Kuchimanchi S, Fitzgerald K, Zimmermann T, van Berkel TJ, Maier MA, rajeev KG, Manoharan M. Multivalent N-acetylgalactosamine-conjugated siRNA localizes in hepatocytes and elicits robust RNAi-mediated gene silencing. J Am Chem Soc 2014; 136: 16958-61.
34 Prakash TP, Graham MJ, Yu J, Carty R, Low A, Chappell A, Schmidt K, Zhao C, Aghajan M, Murray HF, Riney S, Booten SL, Murray SF, Gaus H, Crosby J, Lima WF, Guo S, Monia BP, Swayze EE, Seth PP. Targeted delivery of antisense oligonucleotides to hepatocytes using triantennary Nacetyl galactosamine improves potency 10-fold in mice. Nucleic Acids Res 2014; 42: 8796-807.



3

ACUTE KIDNEY INJURY INDUCED BY PCSK9 TARGETED THERAPY, THE IMPORTANCE OF NOVEL HIGHLY SENSITIVE BIOMARKERS

EP van Poelgeest, RM Swart, MG Betjes, M Moerland, JJ Weening, Y Tessier, MR Hodges, AA Levin, J Burggraaf.
Acute kidney injury during therapy with an antisense oligonucleotide directed against PCSK9.
American Journal of Kidney Disease 2013 Oct;62(4):796-800.

ABSTRACT

Antisense oligonucleotides are widely explored in clinical trials, and generally considered non-toxic for the kidney, even at high concentrations. Here, we report a case of toxic acute tubular injury in a healthy 56 year old female volunteer after administration of a pharmacologically active dose locked nucleic acid antisense oligonucleotide. The patient received 3 weekly subcutaneous doses of experimental drug SPC5001, a Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9) inhibiting antisense oligonucleotide to lower LDL-Cholesterol. Five days after the last dose serum creatinine had increased from 0.81 mg/dL (eGFR: 78 mL/min/1.73 m^2) at baseline to 2.67 mg/dL (eGFR: 20 mL/min/1.73 m^2) and this coincided with the presence of white blood cells, granular casts and minimal hematuria on urine microscopy. Serum creatinine peaked at 3.81 mg/dL (eGFR: 13 mL/min/1.73 m^2) 1 week after the last oligonucleotide dose. Kidney biopsy showed multifocal tubular necrosis and signs of oligonucleotide accumulation. Upon conservative treatment serum creatinine decreased gradually to baseline at 44 days after last oligonucleotide administration. The patient recovered fully and kidney function was normal at every follow-up visit.

INTRODUCTION

Oligonucleotide-based therapies such as antisense-, silencing-, interfering- and micro-RNAs are widely investigated and considered a viable treatment option for a wide variety of clinical conditions [1-3]. Antisense oligonucleotide (ASO) based therapies are most advanced and considered safe [1]. Although clinical experience is limited, oligonucleotides share common features such as their ability to prolong a PTT, elicit injection site skin reactions, and accumulate in kidney proximal tubular cells.

SPC5001 is a short synthetic ASO containing DNA flanked at both ends by locked nucleic acids (LNA), binding to the mRNA of Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9). This protease is expressed primarily in liver, intestine, and kidneys [4-7] and involved in various cellular functions [6;8;9]. The role of PCSK9 in LDL-Receptor degradation renders it a potential target for the treatment of hypercholesterolemia [10;11]. Here, we report on a case of acute tubular injury in a healthy female participating in a first-in-human trial with SPC5001. Other participants treated with at an equal SPC5001 dosage also showed signs of transient tubular dysfunction, suggesting a causal relationship. We aim to increase awareness for the toxic effects of ASOs on the kidney.

CASE REPORT

A 56-year-old caucasian female participated in a first-in-human trial with SPC5001. The patient was healthy as shown by a complete medical screening and did not smoke or use medication. Blood pressure was 126/70 mmHg and ECG and routine laboratory parameters were all normal.

She received 3 sc injections of SPC5001 (5 mg/kg; days 1, 8 and 15). Apart from developing injection site reactions (4x4 cm erythematous spots), there were no remarkable findings up to 24h after the last dose (day 16). At a scheduled assessment at day 18 the patient was feeling well but mentioned she had been nauseous on day 16/17 and had had limited intake of food and fluids. Urine production had been normal and physical examination was unremarkable. Serum creatinine had increased from 0.81 mg/dL at baseline (estimated glomerular filtration rate (eGFR): 78 mL/min/1.73 m^2), to 1.33 mg/dL (eGFR: 44 mL/min/1.73 m^2). At day 20, the patient was still well clinically, but serum creatinine had increased to 2.76 mg/dL (eGFR: 19 mL/min/1.73 m^2) and urine microscopy showed white blood cells, granular casts and minimal hematuria. The patient was admitted and treatment with iv isotonic saline was started. On day 21, serum creatinine

had increased to 3.59 mg/dL (eGFR: 14 mL/min/1.73 m^2), and kidney biopsy was obtained. Histopathology showed several foci of severe tubular injury with total denudation, nuclear apoptosis, and eosinophilic epithelial degeneration with shedding into tubular lumen which also contained debris with granular material (Figure 1). The tubular basal membrane was mostly intact. Intact tubules showed quite high epithelium and a brush border. Glomeruli and vessels were normal; the interstitium showed focal edema and lymphocytic infiltrates. Immunofluorescent staining ruled out immunoglobulin or complement deposition. Electron microscopy showed cell necrosis, vacuolization, and collapse of the cytoskeleton, a picture commonly seen in toxic tubular damage. Electron microscopy also showed abundance of phagolysosomes and endosomes in the affected tubular epithelial cells, reflecting antisense accumulation [13;14]. Without therapeutic intervention, serum creatinine decreased from its peak at 3.81 mg/dL (eGFR: 13 mL/min/1.73 m^2) at day 22, and the patient was discharged at day 24 with 2-weekly return visits scheduled. Serum creatinine decreased further and baseline value 0.88 mg/dL (eGFR: 71 mL/min/1.73 m^2) was reached at day 59. Urine dipstick and microscopy was normal during this follow-up period, except for a single observation of a trace (0-0.3 g/L) of protein at dipstick analysis on day 44. At the final visit at 8 months after the event the patient was well and routine laboratory parameters were normal.

Post-hoc analysis of biobanked spot urine samples, collected before each administration of study medication, was performed for assessment of kidney injury markers β2-microglobulin, α-Glutathione S-Transferase (αGST), Kidney Injury Molecule-1 (KIM1), and N-acetyl-β-D- glucosaminidase (NAG). NAG levels remained unchanged, but urinary β2-microglobulin increased 4-fold, αGST increased 24-fold, and KIM1 increased 60-fold upon administration of SPC5001 (Figure 2). Importantly, these markers preceded the rise in serum creatinine, increasing already after the first SPC5001 administration. These observations suggest that SPC5001 adversely affects proximal tubular function [15;16].

DISCUSSION

We report a case of acute tubular injury observed in a healthy female exposed to the LNA oligonucleotide SPC5001. Kidney toxicity became apparent within a week after the third and final dose, suggesting causal relationship. The toxicity was first noted by increases in serum creatinine, but was in hindsight preceded by increases in αGST, KIM1, and β2-microglobulin after the first dose implying that proximal tubular dysfunction was already present before the clinical manifestation

or routine laboratory measures increased. The causal relationship with the drug is supported by observations in other healthy volunteers treated with the same SPC5001 dosage in whom in the week after the final dose, transient but less pronounced (~15%) increases in serum creatinine, urinary damage markers and casts (3 out of 5 subjects) were noted.

Antisense compounds generally behave similarly with regard to kidney accumulation. Among the different chemistries of ASOs, 2'-O methoxyethyl modified phosphorothioate moieties (MOE-ASO) and 2'-4' O methylene LNAs are most researched and have been shown to accumulate without causing functional impairment even at dosages above those required for pharmacologic activity and during dehydration in animals [17]. Indeed, SPC5001- uptake, as with other oligonucleotides [18], is most prominent in the proximal tubule, but no adverse biochemical or histological kidney effects were noted in non-human primates after a loading dose of 20 mg/kg followed by 4 weekly doses of 5 mg/kg [19]. Dosages of this magnitude with other oligonucleotides did not induce cell toxicity and loss of renal function [20;21].

However, it has been reported that ASOs may concentration-dependently affect tubular cells [17], and that (repeated) exposure to high ASO doses may result in kidney toxicity [20;22-24]. This could reflect lysosomal stress and downstream apoptosis due to oligonucleotide accumulation in proximal tubule cells that are highly metabolically active to maintain integrity/function [25].

ATN associated with long term (74 doses of 10mg/kg weekly) MOE-ASO treatment has been described to occur in a cancer patient [26], but we now show that also 3 weekly doses of an LNA-ASO in healthy subjects may have caused acute renal injury. The MOE-ASO and LNA-ASO clinical case have in common that the target of the oligonucleotide is expressed in the kidney. Differences include the general condition of the patients (healthy vs. metastatic melanoma), the cumulative dose (1050 vs. 55500 milligram), the time course of recovery (1.5 vs. 4 months), and the time of the biopsy (1 wk vs. 4 months after final dose).

The precise mechanism by which SPC5001 caused kidney injury could have been target-related as PCSK9 is expressed in the kidney [27;28] and upregulation of renal PCSK9 mRNA occurs during inflammation [29], probably as a mechanism by which kidney injury is mitigated in a mouse model of drug-induced renal toxicity [30]. However, a direct effect of PCSK9-inhibition on the kidney seems at odds with the data showing that a novel interfering RNA inhibited PCSK9 by 60% without any effect on renal function [31], and loss of function mutations seem unassociated with impaired kidney function [32]. The oligonucleotide exposure in our SPC5001-treated patient is in line with exposure levels of previously studied

ASOs [33;34], and the urinary excretion was comparable to the other volunteers making renal toxicity due to excessively high local SPC5001 exposure unlikely. All observations point into the direction that renal oligonucleotide accumulation may have untoward effects in susceptible subjects. It is conceivable that susceptibility to develop toxic kidney disease varies between subjects. It may have been that our patient was predisposed to develop overt tubulotoxicity, as genetic differences affecting intracellular transport proteins and drug efflux transporters have been described [35]. Although the exact cause of the observed renal toxicity remains uncertain, and there may be differences between ASOs based on their chemistry, on- or off-target pharmacology and possibly other factors [1], we advocate careful monitoring of kidney function in the clinical development and utilization of ASOs. This should include not only serum creatinine or blood urea nitrogen (BUN), but also urine microscopy [36] and specific renal damage markers. With this approach, earlier detection and cessation of treatment is possible without relying solely on traditional markers for renal function such as creatinine, BUN, and electrolytes, particularly in subjects with uncompromised kidney function in whom early signs of toxic kidney disease might be masked [37]. These markers may also provide important mechanistic insight into the nature of potential renal damage.

Figure 1A&B. Light micrograph of the renal cortex at original magnification (A) x40 and (B) x80. Approximately 1 cm of kidney cortex with 21 glomeruli was viewed by light microscopy; ~20% of the tubules showed severe tubular necrosis (**A-B**). One glomerulus in the renal cortex is displayed showing several foci of severe tubular necrosis (*) with total denudation, vacuolization with loss of cytoplasm, nuclear apoptosis, and eosinophilic epithelial degeneration with shedding into tubular lumina, which contained debris with granular material (arrow). The tubular basal membrane was mostly intact (**A**). Glomeruli and vascular architecture showed no abnormalities, and interstitial tissue showed some edema and patchy lymphocytic infiltrates. (**B**) *(See figure 1C&D on next page).*

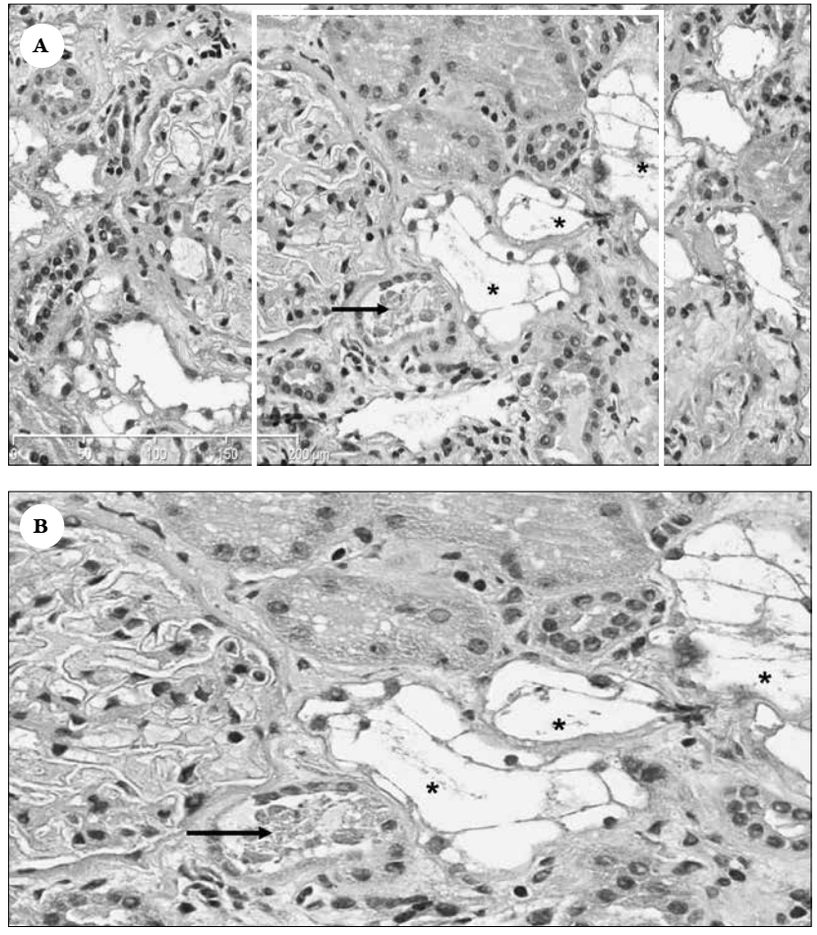
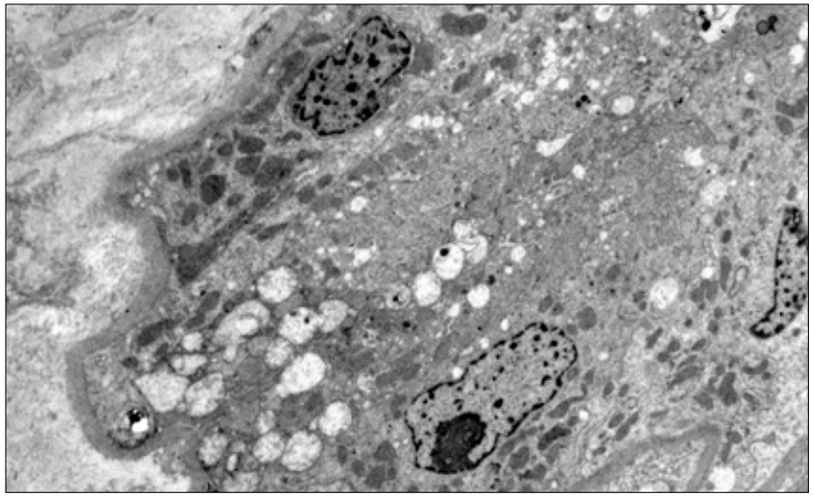


Figure 1C&D. Electron microscopy detail of proximal tubule cell. Electron microscopy of tubular cells shows increased endosomes (C) and clear nuclear condensation with increased lysosomes between swollen mitochondria (D). *(See figure 1A&B on previous page).*



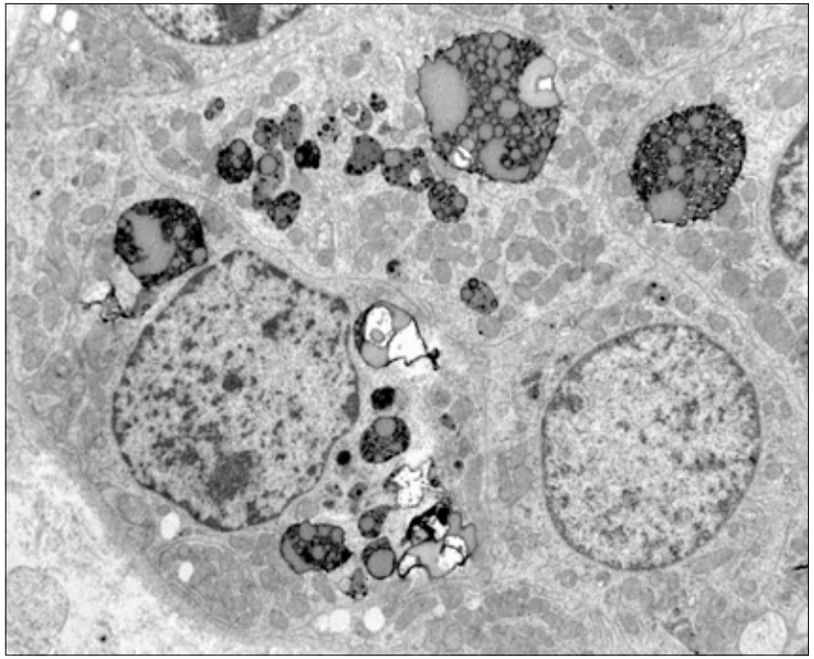
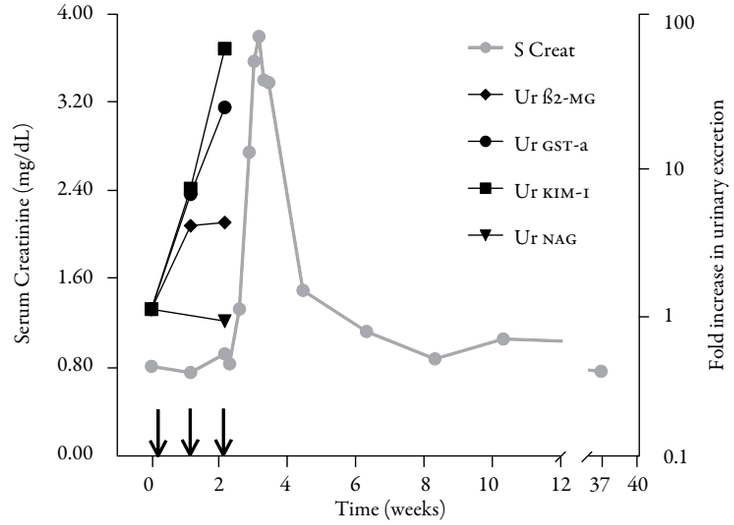


Figure 2. Time course of serum creatinine (S Creat) and urinary kidney damage marker levels. Arrows denote administration of SPC5001 on study days 1, 8, and 15. Conversion factor for S Creat in mg/dL to µmol/L, x88.4.



Abbreviations: Ur β2MG, urinary β2-microglobulin; Ur GSTα, urinary α-glutathione *S* transferase; Ur KIM1, urinary kidney injury molecule 1; Ur NAG, urinary *N*-acetyl-β-D-glucosaminidase.

REFERENCES

1 Bennett CF, Swayze EE. RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. Annu Rev Pharmacol Toxicol 2010;50:259-293.

2 Lippi G, Favaloro EJ. Antisense therapy in the treatment of hypercholesterolemia. eur J Intern Med 2011;22(6):541-546.

3 rayburn ER, Zhang R. Antisense, RNAi, and gene silencing strategies for therapy: mission possible or impossible? Drug Discov Today 2008;13(11-12):513-521.

4 Cohen JC, Boerwinkle E, Mosley TH, Jr., Hobbs HH. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. N Engl J Med 2006;354(12):1264-1272.

5 Luo Y, Warren L, Xia D et al. Function and distribution of circulating human PCSK9 expressed extrahepatically in transgenic mice. J Lipid Res 2009;50(8):1581-1588.

6 Cariou B, Le MC, Costet P. Clinical aspects of PCSK9. Atherosclerosis 2011;216(2):258-265.

7 Lambert G, Krempf M, Costet P. PCSK9: a promising therapeutic target for dyslipidemias? Trends Endocrinol Metab 2006;17(3):79-81.

8 Tibolla G, Norata GD, Artali R, Meneghetti F, Catapano AL. Proprotein convertase subtilisin/kexin type 9 (PCSK9): from structure-function relation to therapeutic inhibition. Nutr Metab Cardiovasc Dis 2011;21(11):835-843.

9 Horton JD, Cohen JC, Hobbs HH. Molecular biology of PCSK9: its role in LDL metabolism. Trends Biochem Sci 2007;32(2):71-77.

10 Fahed AC, Nemer GM. Familial hypercholesterolemia: the lipids or the genes? Nutr Metab 2011;8(1):23.

11 Sjouke B, Kusters DM, Kastelein JJ, Hovingh GK. Familial hypercholesterolemia: present and future management. Curr Cardiol Rep 2011;13(6):527-536.

12 Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. Ann Intern Med 1999;130(6):461-470.

13 Henry SP, Templin MV, Gillett N, Rojko J, Levin AA. Correlation of toxicity and pharmacokinetic properties of a phosphorothioate oligonucleotide designed to inhibit ICAM-1. Toxicol Pathol 1999;27(1):95-100.

14 Butler M, Stecker K, Bennett CF. Cellular distribution of phosphorothioate oligodeoxynucleotides in normal rodent tissues. Lab Invest 1997;77(4):379-388.

15 Bonventre JV, Vaidya VS, Schmouder R, Feig P, Dieterle F. Next-generation biomarkers for detecting kidney toxicity. Nat Biotechnol 2010;28(5):436-440.

16 Perazella MA, Coca SG. Traditional urinary biomarkers in the assessment of hospital-acquired aki. Clin J Am Soc Nephrol 2012;7(1):167-174.

17 Henry SP, Johnson M, Zanardi TA et al. Renal uptake and tolerability of a 2'-O-methoxyethyl modified antisense oligonucleotide (ISIS 113715) in monkey. Toxicology 2012;301(1-3):13-20.

18 van de Water FM, Boerman OC, Wouterse AC, Peters JG, Russel FG, Masereeuw R. Intravenously administered short interfering RNA accumulates in the kidney and selectively suppresses gene function in renal proximal tubules. Drug Metab Dispos 2006;34(8):1393-1397.

19 Lindholm MW, Elmen J, Fisker N et al. PCSK9 LNA antisense oligonucleotides induce sustained reduction of LDL cholesterol in nonhuman primates. Mol Ther 2012;20(2):376-381.

20 Monteith DK, Horner MJ, Gillett NA et al. Evaluation of the renal effects of an antisense phosphorothioate oligodeoxynucleotide in monkeys. Toxicol Pathol 1999;27(3):307-317.

21 Lindow M, Kauppinen S. Discovering the first microRNA-targeted drug. J Cell Biol 2012;199(3):407-412.

22 Henry SP, Bolte H, Auletta C, Kornbrust DJ. Evaluation of the toxicity of ISIS 2302, a phosphorothioate oligonucleotide, in a four-week study in cynomolgus monkeys. Toxicology 1997;120(2):145-155.

23 Srinivasan SK, Iversen P. Review of *in vivo* pharmacokinetics and toxicology of phosphorothioate oligonucleotides. J Clin Lab Anal 1995;9(2):129-137.

24 Sarmiento UM, Perez JR, Becker JM, Narayanan R. In vivo toxicological effects of rel A antisense phosphorothioates in CD-1 mice. Antisense Res Dev 1994;4(2):99-107.

25 Ferguson MA, Vaidya VS, Bonventre JV. Biomarkers of nephrotoxic acute kidney injury. Toxicology 2008;245(3):182-193.

26 Herrington WG, Talbot DC, Lahn MM et al. Association of long-term administration of the survivin mRNA-targeted antisense oligonucleotide LY2181308 with reversible kidney injury in a patient with metastatic melanoma. Am J Kidney Dis 2011;57(2):300-303.

27 Seidah NG, Mayer G, Zaid A et al. The activation and physiological functions of the proprotein convertases. Int J Biochem Cell Biol 2008;40(6-7):1111-1125.

28 Ferri N, Tibolla G, Pirillo A et al. Proprotein convertase subtilisin kexin type 9 (PCSK9) secreted by cultured smooth muscle cells reduces macrophages LDL-R levels. Atherosclerosis 2012;220(2):381-386.

29 Feingold KR, Moser AH, Shigenaga JK, Patzek SM, Grunfeld C. Inflammation stimulates the expression of PCSK9. Biochem Biophys Res Commun 2008;374(2):341-344.

30 Fujiwara Y, Tsuchiya H, Sakai N, Shibata K, Fujimura A, Koshimizu TA. Proximal tubules and podocytes are toxicity targets of buciLlamine in a mouse model of drug-induced kidney injury. eur J Pharmacol 2011;670(1):208-215.

31 Fitzgerald K, Frank-Kamenetsky M, Mant T et al. Phase I safety, pharmacokinetic, and pharmacodynamic results for ALN-PCS, a novel RNAi therapeutic for the treatment of hypercholesterolemia. Arteriosclerosis, Thrombosis, and Vascular Biology 2012;32(Supplement 5 (Meeting Abstracts)):A67.

32 Zhao Z, Tuakli-Wosornu Y, Lagace TA et al. Molecular characterization of loss-of-function mutations in PCSK9 and identification of a compound heterozygote. Am J Hum Genet 2006;79(3):514-523.

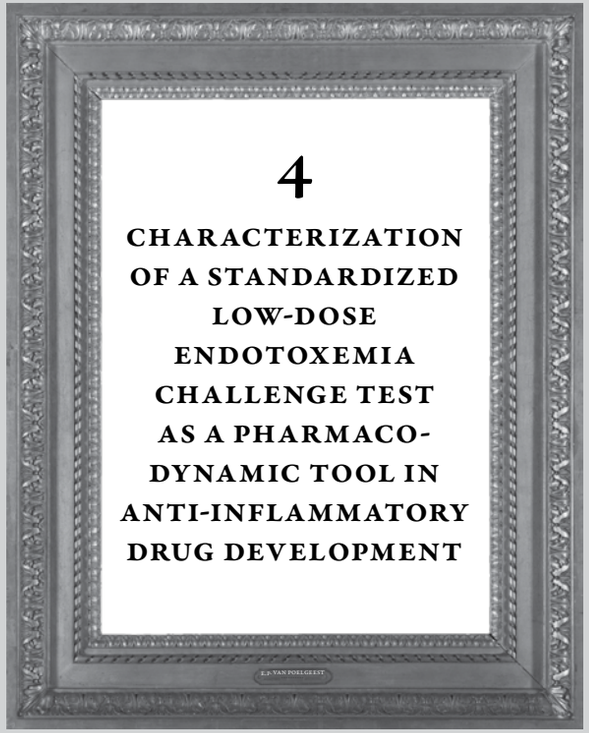
33 Akdim F, Stroes ES, Sijbrands EJ et al. Efficacy and safety of mipomersen, an antisense inhibitor of apolipoprotein B, in hypercholesterolemic subjects receiving stable statin therapy. J Am Coll Cardiol 2010;55(15):1611-1618.

34 Krieg AM. Targeting LDL Cholesterol With LNA. Mol Ther Nucleic Acids 2012;1:e6.

35 Perazella MA. Toxic nephropathies: core curriculum 2010. Am J Kidney Dis 2010;55(2):399-409.

36 Hall IE, Coca SG, Perazella MA et al. Risk of poor outcomes with novel and traditional biomarkers at clinical AKI diagnosis. Clin J Am Soc Nephrol 2011;6(12):2740-2749.

37 Waring WS, Moonie A. Earlier recognition of nephrotoxicity using novel biomarkers of acute kidney injury. Clin Toxicol (Phila) 2011;49(8):720-728.



4

CHARACTERIZATION OF A STANDARDIZED LOW-DOSE ENDOTOXEMIA CHALLENGE TEST AS A PHARMACO-DYNAMIC TOOL IN ANTI-INFLAMMATORY DRUG DEVELOPMENT

MR Dillingh*, EP van Poelgeest*, KE Malone, EM Kemper, ESG Stroes, M Moerland. *Characterization of inflammation and immune cell modulation induced by low-dose LPS administration to healthy volunteers*. Journal of Inflammation 2014, 11:28. (* Equal contribution)

ABSTRACT

BACKGROUND Human *in vivo* models of systemic inflammation are used to study the physiological mechanisms of inflammation and the effect of drugs and nutrition on the immune response. Although *in vivo* lipopolysaccharide (LPS) challenges have been applied as methodological tool in clinical pharmacology studies, detailed information is desired on dose-response relationships, especially regarding LPS hyporesponsiveness observed after low-dose *in vivo* LPS administration. A study was performed to assess the *in vivo* inflammatory effects of low intravenous LPS doses, and to explore the duration of the induced LPS hyporesponsiveness assessed by subsequent *ex vivo* LPS challenges.

METHODS This was a randomized, double-blind, placebo-controlled study with single ascending low doses of LPS (0.5, 1 and 2 ng/kg body weight) administered to healthy male volunteers (3 cohorts of 8 subjects, LPS:placebo 6:2). The *in vivo* inflammatory response was assessed by measurement of cytokines and CRP. *Ex vivo* LPS challenges were performed (at -2, 6, 12, 24, 48 and 72 hours relative to *in vivo* LPS administration) to estimate the duration and magnitude of LPS hyporesponsiveness by assessment of cytokine release (TNFα, IL1β, IL6, IL8).

RESULTS LPS administration dose-dependently increased body temperature (+1.5 °C for 2 ng/kg LPS), heart rate (+28 bpm for 2 ng/kg LPS), CRP and circulating cytokines which showed clearly distinctive increases from placebo already at the lowest LPS dose level tested (0.5 ng/kg, contrast for timeframe 0-6 hours: TNFα +413%, IL6 +288%, IL8 +254%; all p≤0.0001). *In vivo* LPS administration dose-dependently induced a period of hyporesponsiveness in the *ex vivo* LPS-induced cytokine release (IL1β, IL6 and TNFα), with maximal hyporesponsiveness observed at 6 hours, lasting no longer than 12 hours. For IL6 and IL8, indications for immune cell priming were observed.

CONCLUSIONS We demonstrated that an *in vivo* LPS challenge, with LPS doses as low as 0.5 ng/kg, elicits a cytokine response that is clearly distinctive from baseline cytokine levels. This study expanded the knowledge about the dose-effect relationship of LPS-induced hyporesponsiveness. As such, the low-dose LPS challenge has been demonstrated to be a feasible methodological tool for future clinical studies exploring pharmacological or nutritional immune-modulating effects.

INTRODUCTION

Human models of systemic inflammation have been developed with the purpose to explore the molecular mechanisms and physiological significance of the systemic inflammatory response encountered in acute as well as chronic inflammatory conditions, such as sepsis, trauma, type 2 diabetes, atherosclerosis, and Alzheimer's disease, in a controlled, standardized experimental setting. A better understanding of the underlying molecular and pathophysiological mechanisms could lead to optimized prevention and treatment of these disorders, associated with morbidity and mortality [1]. In addition, human models of systemic inflammation can be applied in clinical pharmacology studies to assess the effects of specific interventions (medicinal or non-medicinal) on the inflammatory response in non-diseased populations.

Human endotoxemia is often used as a model of systemic inflammation. In this experimental setting, purified lipopolysaccharide (LPS, also referred to as endotoxin) from the cell membrane of *Escherichia coli* (*E.coli*) or other Gram-negative bacteria is administered intravenously to healthy volunteers resulting in flu-like symptoms, increased production of C-reactive protein (CRP) and increased concentrations of pro- and anti-inflammatory cytokines. Since the effects of *E.coli* are highly reproducible, this is the predominant bacterial source used [1]. LPS induces an inflammatory response via stimulation of Toll-like receptors (TLRs), basic signaling receptors of the innate immune system activated by tissue damage or by molecules associated with pathogen-associated molecular patterns (PAMPs) on invading microorganisms. LPS is known to activate multiple intracellular pathways (e.g. the MYD88-dependent and TRIF-dependent pathways) [2,3].

The human endotoxemia model has been studied extensively, and commonly applies relatively high LPS doses (2-4 ng/kg body weight) [1,4-8]. However, an endotoxemia model applying such relatively high LPS doses is not preferred as methodological tool in clinical pharmacology studies since the elicited immune response is so strong that potential effects of immune-modulating interventions may not be observed, other homeostatic mechanisms may be temporarily impaired, and, importantly, the elicited immune response at these LPS doses is not free of risk for the volunteer. Studies applying lower LPS doses have been performed [9,10], but thorough characterization of a human endotoxemia model at lower LPS dose levels is desired. In the current study, 0.5 ng/kg was selected as the lowest LPS dose to be administered intravenously because an LPS dose of 0.2 ng/kg was shown previously to elicit no cytokine response *in vivo* [11]. We performed a study to characterize the LPS dose relationship of the human inflammatory

response at low LPS doses (0.5, 1 and 2 ng/kg) administered to healthy volunteers. Furthermore, we explored the effects of such an *in vivo* LPS challenge on the inflammatory response induced by subsequent *ex vivo* LPS challenges. It has been described that an *in vivo* LPS challenge induces hyporesponsiveness to following *in vivo* or *ex vivo* LPS challenges. The biochemical mechanisms accounting for this hyporesponsiveness have been demonstrated to involve negative regulators such as IRAK-M, SOCS-1, SHIP, ST2 and IL10 [2,3,12-18] and downregulation of CD14 [19].

It has been reported that *ex vivo* LPS hyporesponsiveness following an *in vivo* LPS challenge was resolved after 1 week [4]. However, the exact time course of this phenomenon and relation to LPS dose level is unclear. Since such information could be important for (repeated) application of *in vivo* and *ex vivo* LPS challenges in clinical pharmacology studies, characterization of this hyporesponsiveness was an objective of our study.

METHODS

The study was conducted in accordance with the Declaration of Helsinki and Guideline for Good Clinical Practice, and was approved by the Medical Ethics Review Board of the Academic Medical Center, Amsterdam, The Netherlands.

SUBJECTS Twenty-four healthy male volunteers, aged 18-28 years (inclusive) with a BMI of 18 to 25 kg/m^2 and a body weight ≥ 56 kg, participated in this study. After providing informed consent, subjects were medically screened within 3 weeks prior to participation. Exclusion criteria included history of sepsis, cardiovascular disease, previous syncope or malignancy, hemorrhagic diathesis, any active inflammatory or infectious disease, renal impairment, diabetes mellitus, thyroid dysfunction, and prior exposure to endotoxin in an experimental setting within 4 weeks of the anticipated exposure. Any use of medication that in the opinion of the investigator would complicate or compromise the study or interfere with the study objectives was not permitted during the study.

STUDY DESIGN This was a randomized, blinded, placebo controlled study of ascending single doses of 0.5, 1 and 2 ng/kg LPS (U.S. Reference *Escherichia Coli* (*E.coli*) endotoxin CC-RE-Lot 3 (O113:H, 10:K negative, National Institute of Health, Bethesda, MD, US, approximately 10 eu/ng; or placebo), administered to healthy male subjects as an intravenous bolus over 2 minutes. Each cohort included eight healthy subjects, of which six subjects received LPS and two placebo (sodium chloride 0.9%). Subjects were prehydrated with 1500 mL glucose/saline (2.5% glucose/0.45% sodium chloride) 2 hours prior to LPS (/placebo) administration, followed by an intravenous drip of 150 mL/hr for a period of 6 hours. After LPS/placebo administration, subjects were confined to the clinical research unit for 24 hours.

SAFETY MONITORING Safety monitoring was performed by adverse events monitoring, physical examination, assessment of electrocardiogram (ECG) and vital signs, and laboratory evaluations (routine hematology, chemistry, coagulation, and semi-quantitative dipstick urinalysis). In case of clinically significant findings in dipstick analysis, a microscopic investigation of the urine was performed. For subject safety, maximally two subjects were treated within one day, with a lag time of at least 40 minutes between subjects. All blinded safety data collected up to at least 24 hours after LPS/placebo administration were reviewed before the decision was made to escalate the LPS dose level and proceed with the next cohort.

INFLAMMATORY MARKERS The systemic inflammatory response was assessed by frequent measurement of C-reactive protein (CRP) and a panel of cytokines (IL1β, IL6, IL8 and TNFα) using a human ultra-sensitive 4-plex (MSD). CRP levels were measured as part of the standard chemistry panel. Samples for cytokine analysis were collected in sodium heparin (Greiner) tubes. In addition, the effect of an *in vivo* LPS challenge on cytokine release (IL1β, IL6, IL8, TNFα) induced by an *ex vivo* LPS challenge was studied. Blood samples were collected in sodium heparin tubes (Greiner) before and 6, 12, 24, 48 and 72 hours after the *in vivo* LPS administration. Whole blood cultures were prepared with a 1:1 dilution with RPMI 1640 medium and incubated with LPS (*E.coli* O111:B4, manufactured by Sigma-Aldrich, Saint Louis, MO, US, catalog number L-3012, approximately 10 eu/ng) for 24 hours at 37 °C, 5% CO2. Cultures were centrifuged and supernatants were used for cytokine assessment using the earlier mentioned cytokine 4-plex with a 20-fold dilution. Whole blood cultures were performed by Good Biomarker Sciences, Leiden, The Netherlands.

STATISTICAL ANALYSIS Statistical analysis was performed for circulating inflammatory markers and *ex vivo*-induced cytokines, which were log-transformed prior to analysis. These repeatedly measured parameters were analyzed with a mixed model of variance with treatment, time, and treatment by time as fixed factors and subject as random factor and the baseline measurement as covariate. A variance components (co-) variance structure was used to model the within-subject errors, the Kenward-Roger approximation to estimate denominator degrees of freedom and the restricted maximum likelihood method to estimate model

parameters. Contrasts were calculated within the model for each parameter over the following time profiles: baseline to 6 hours post-dose for circulating cytokines; baseline to 24 hours post-dose for CRP; and at 6 hours post-dose for *ex vivo*-induced cytokines. The general treatment effect and specific contrasts were reported with the estimated difference, the 95% confidence interval (CI), the least square mean (LSM) estimates and the p-value. Graphs of the LSMs estimates over time by treatment present 95% confidence intervals as error bars and change from baseline LSMs estimates. All analyses were performed using SAS for Windows Version 9.1.3 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

SAFETY Single intravenous low doses of LPS were well tolerated in healthy male subjects. Observed adverse events (AEs) were of mild severity and self-limiting without therapeutic intervention. The most frequent occurring AEs, probably or possibly related to treatment, were headache, observed in 66.7% of the LPS-treated subjects and 33.3% of the placebo-treated subjects and feeling cold, observed in 44.4% of the LPS-treated subjects and none of the placebo-treated subjects. No clinically relevant changes or unexpected treatment-related trends were observed in supine systolic and diastolic blood pressure, body temperature, or ECG-derived parameters following administration of LPS (Figure 1). LPS dose-dependently increased body temperature and heart rate, with a maximal increase amounting approximately 1.5 °C and 28 ± 13.2 bpm for the highest LPS dose tested, observed at 3-4 hours after LPS administration.

LPS administration resulted in a dose-dependent decrease in monocyte count (maximal change from baseline -1.9 ± 2.3, -4.8 ± 3.6 and -7.7 ± 1.5% at 6 hours post-LPS for doses of 0.5, 1 and 2 ng/kg, respectively), returning to baseline levels within 12 to 24 hours post-LPS (data not shown). In addition, LPS administration resulted in decreased blood platelet count levels (minimal change from baseline of -15 ± 9.4, -28 ± 14.4, and -31 ± 9.1*10^9/L at 4 hours post-dose, data not shown) and an increase in neutrophil count (maximum change from baseline 25.8 ± 3.5, 40.4 ± 9.5, and 42.9 ± 6.2% at 4 hours post-LPS for doses of 0.5, 1 and 2 ng/kg, respectively) and leukocyte count (maximum change from baseline 4.0 ± 1.3, 4.4 ± 1.2, 6.3 ± 1.4*10^9/L at 4-6 hours post-LPS), returning to baseline at 12-24 hours post-dose (data not shown). Eosinophil, erythrocyte, lymphocyte, and basophil counts and hematocrit and hemoglobin slightly decreased after LPS administration, with maximal changes observed 4 hours after LPS administration (data not shown).
Activated partial thromboplastin time (aPTT) was variable over the day, ranging from -0.7 to +0.9 s around a baseline concentration of 29.3 ± 1.8 s. LPS administration resulted in a decrease in aPTT, with an estimated difference of -2.0 s (p=0.0151) at 1 ng/kg LPS and a maximal mean decrease from baseline of -3.9 s at 4 hours post-LPS (Figure 2). Although the decrease in aPTT upon administration of 2 ng/kg LPS was comparable in size, this difference did not reach a level of statistical significance (p=0.1233). An effect of LPS administration on prothrombin time (PT) was not observed (data not shown).

CIRCULATING INFLAMMATORY MARKERS CRP levels were low in the placebo-treated group (data not shown; baseline concentration 0.85 ± 1.08 mg/L, with a minimal variability over time of maximally 0.30 ± 0.55 mg/L. LPS administration dose-dependently increased CRP, maximal levels observed 24 hours post-dose (11.31 ± 6.73, 15.15 ± 3.93, and 18.42 ± 5.15 mg/L for LPS doses of 0.5, 1 and 2 ng/kg, respectively, Figure 3A; all contrasts presented for the complete time profile up to 24 hours post dose, versus placebo, p<0.0001). In the placebo-treated group, circulating cytokine levels were minimal (Figure 3B-D). LPS administration resulted in a dose-dependent increase in TNFα, IL6, and IL8, with maximal levels amounting 221.9 ± 61.2 pg/mL, 314.8 ± 130.9 and 329.4 ± 84.4 pg/mL, respectively. Maximal concentrations were reached at 1.5-3 hours after LPS administration. For all LPS dose levels tested, contrasts for cytokine release versus placebo (time interval 0-6 hours post-dose) reached a distinct level of significance (p<0.0001). In a considerable number of samples, IL1β levels were below the limit of quantification (LOQ, 0.6 pg/mL, data not shown). In general, higher levels of IL1β were observed with increasing LPS doses. For all subjects in the 2 ng/kg dose group, IL1β levels above LOQ could be detected 3-6 hours post-LPS, ranging from 0.7 to 2.6 pg/mL. No statistical analysis was performed for IL1β.

EX VIVO LPS-INDUCED CYTOKINE RELEASE *Ex vivo* LPS-induced IL1β, IL6, IL8 and TNFα release was variable over time, as observed in the placebo-treated subjects (Figure 4). *In vivo* LPS administration dose-dependently decreased *ex vivo* LPS-induced TNFα release at the highest two dose levels tested in the first hours after the *in vivo* LPS challenge (Figure 4A). Maximal mean reduction was observed at 6 hours post-dose with an estimated difference (95% CI) of -66.4% (-81.4 to -39.0%) and -74.7% (-86.0 to -54.3%) for 1 and 2 ng/kg, respectively, which differed significantly from placebo (Table 1, p=0.0005 and p<0.0001 for 1 and 2 ng/kg, respectively). Subsequently, TNFα release increased and exceeded levels as observed for the placebo group at 12 hours post-LPS. *Ex vivo* LPS-induced IL1β release basically mirrored the patterns observed for TNFα release, with a

maximal mean reduction at 6 hours post-LPS with an estimated difference (95% CI) of -65.8% (-79.5 to -43.1%) and -84.7% (-90.8 to -74.5%; Figure 4B and Table 1; p<0.0001 and p<0.0001 versus placebo for 1 and 2 ng/kg, respectively), and a return to placebo levels at approximately 12 hours post-dose. An *in vivo* LPS challenge at 1 and 2 ng/kg significantly inhibited the *ex vivo* LPS-induced release of IL6 lasting for approximately 12 hours post-LPS and a maximal effect at 6 hours with an estimated difference (95% CI) of -31.3 % (-50.8 to -4.0%) and -41.3% (-58.1 to -17.8%; Figure 4C and Table 1; p=0.0283 and p=0.0024 versus placebo for 1 and 2 ng/kg). Remarkably, *ex vivo* IL6 release increased after the *in vivo* administration of 0.5 ng/kg LPS, peaking at 6 hours post-LPS and almost significantly exceeding cytokine levels observed for placebo-treated subjects with an estimated difference (95% CI) of 34.8% (-3.1 to 87.5% (Figure 4C and Table 1; p=0.0754 versus placebo). A same response was observed for *ex vivo* IL8 release: *in vivo* administration of LPS resulted in an increased IL8 response to an *ex vivo* LPS challenge for the two lowest LPS doses tested with an estimated difference (95% CI) of 55.1% (-6.6 to 157.5%) and 19.2% (-28.8 to 99.5%) (Figure 4D and Table 1; at 6 hours, p=0.0879 and p=0.4961 versus placebo, for 0.5 and 1 ng/kg), but not for the 2 ng/kg dose. The observed increases in IL8 release were followed by a strong decrease up to 12 hours post-dose, which was consistent for all LPS doses tested (Figure 4D).

DISCUSSION

Human endotoxemia has been applied frequently as a controlled and standardized model of systemic inflammation providing mechanistic insight in molecular and physiological inflammatory pathways. The *in vivo* LPS challenge can also be applied as methodological tool in clinical pharmacology studies to assess the effects of specific interventions (medicinal or non-medicinal) on the inflammatory response in healthy volunteers. This experimental model has been studied extensively, and commonly applies relatively high LPS doses (2-4 ng/kg bodyweight) [1,4-8]. However, such relatively high LPS doses are not preferred for reasons mentioned, and characterization of a human endotoxemia model applying lower LPS dose levels is desired. Therefore, we performed a study to characterize the human inflammatory response induced by low LPS doses administered to healthy volunteers. In addition, we explored the effects of an *in vivo* LPS challenge on the inflammatory response induced by subsequent *ex vivo* LPS challenges. Although it is known from literature that *in vivo* LPS challenge induces hyporesponsiveness to subsequent *in vivo* or *ex vivo* LPS challenges [2,3,12-18], the exact time course of this phenomenon and relation to LPS dose level is unclear.

Administration of low LPS doses (0.5-2 ng/kg) to healthy volunteers was well-tolerated and safe; all reported AEs were of mild severity and self-limiting, and no unexpected treatment-related trends in vital signs or ECG recordings nor in urinary or blood laboratory parameters were measured. LPS administration dose-dependently increased body temperature and heart rate with maximum levels observed at 3-4 hours post-dose (change to baseline of approximately 1.5 °C and 28 ± 13.2 bpm). Observed changes in hematology parameters were expected as a result of LPS treatment [5,8,20,21] and subject hydration from 2 hours pre-dose till 6 hours post-dose. Furthermore, LPS administration temporarily inhibited aPTT, with a maximal decrease from baseline of approximately 3-4 s at 4 hours post-LPS, in line with previously reported LPS effects on coagulation [22,23]. There is a close interaction between coagulation and inflammation pathways [24,25]. Stimulation of monocytes with endotoxin results in an increased expression of tissue factor, the main initiator of coagulation [23,26,27]. Based on this observation an increase in PT levels was expected via the extrinsic pathway, however, this could not be confirmed by the results from our study due to a high variability over the 24hr time profile. Cytokines such as IL6 and TNFα are the main mediators of inflammation-induced coagulation [28,29]. Whereas changes in temperature and heart rate after an *in vivo* LPS challenge could serve as a pharmacodynamic readout measure for pharmacological or dietary interventions, aPTT effect size and contrast versus placebo of an *in vivo* LPS challenge were limited.

In vivo LPS administration dose-dependently increased circulating CRP and cytokine levels (TNFα, IL6 and IL8). Maximal CRP levels were observed 24 hours post-LPS and maximal cytokine levels were observed 1.5-3 hours post-LPS. A single intravenous dose of LPS as low as 0.5 ng/kg induced a distinct inflammatory response in the healthy volunteers.

A power calculation was performed which showed that in a parallel study design, at an LPS dose level of 0.5 ng/kg, a sample size of 8 subjects per treatment group would provide 80% power to detect an 28% inhibition in the LPS-induced TNFα response, at a two-sided significance level of 0.05. Under the same conditions, it would be possible to demonstrate an inhibition of the LPS-induced cytokine response of 53% and 49% for IL6 and CRP, respectively. Given the fact that the inter subject variability on log scale is well comparable between different LPS doses, this power calculation also applies for LPS doses of 1 and 2 ng/kg.

Circulating IL1β levels were low and for the majority of the samples tested the level was below LOQ (0.6 pg/mL). However, in the highest LPS dose group tested, an increase in circulating IL1β levels could be demonstrated at 3-6 hours post-LPS, with observed IL1β levels ranging from 0.7 to 2.6 pg/mL. This is in contrast with

IL1β release following an *ex vivo* LPS challenge of whole blood cultures, which caused the release of substantial amounts of IL1β. Reports from other human endotoxemia experiments also note IL1β responses are very low or lacking, despite high circulating levels of IL6 and TNFα [30-35]. Interestingly, even in cases of severe sepsis, IL1β can be detected in only a small fraction of patients and corresponds weakly with disease severity [36]. However it can be acutely induced in response to certain surgical procedures, and is implicated in many chronic inflammatory conditions, such as diabetes, cardiovascular disease, and rheumatoid arthritis. Since IL1β expression is limited to inflammasome activation and requires multiple signals [37,38], this suggests that low-dose human endotoxemia may be insufficient to induce systemic IL1β. LPS stimulation in whole blood cultures also induces cell death, which may facilitate inflammasome activation and induce substantial IL1β release as seen in our *ex vivo* LPS experiment.

In vivo LPS administration induced LPS hyporesponsiveness as evidenced by *ex vivo* cytokine release of IL1β, IL6 and TNFα. This hyporesponsiveness was LPS dose-dependent. Although the kinetics of endotoxin hyporesponsiveness have been described previously, the exact time course of the hyporesponsiveness is not well documented [4]. Here we demonstrate that LPS-induced hyporesponsiveness of specific cytokines reached a maximum at 6 hours after the *in vivo* LPS challenge, and lasted no longer than 12 hours. Interestingly, it has been reported that attenuated cytokine responses *in vivo* persisted for at least 2 weeks [4]. This indicates that there is a significant discrepancy between the LPS hyporesponsiveness measured after an *in vivo* LPS challenge, for which the tissue-resident macrophages, migrating leukocytes and endothelial cells are implicated to be the main sources of cytokine production, and an *ex vivo* LPS challenge, for which only the circulating leukocytes are the source of cytokine release and there is no active clearance of endotoxin since the system is closed [4,39]. The fact that the estimated duration of the derangement of the immune system induced by an *in vivo* LPS challenge is dependent on the selected methodology (assessment by *ex vivo* LPS challenge or *in vivo* LPS challenge) should be carefully taken into account when designing future clinical pharmacology studies applying *in vivo*/*ex vivo* LPS challenges, and this process should be driven by the nature and mechanism of action of the investigational product.

Interestingly, patterns for *ex vivo* LPS-induced IL8 release (at all *in vivo* LPS doses tested) and IL6 release (at the lowest *in vivo* LPS dose tested) differed from the patterns observed for IL1β and TNFα: a preceding *in vivo* LPS challenge caused an increased cytokine release after an *ex vivo* LPS challenge, rather than an inhibition of cytokine release. It may be well possible that immune cells were primed by the low-dose *in vivo* LPS challenge, resulting in an augmented IL8 and IL6 responses after *ex vivo* LPS stimulation. Priming of innate immune cells by low endotoxin levels has been described before, and allows the immune system to elicit a strong inflammatory response against potential pathogens [2]. Although priming of the murine immune system has been explored rather extensively, the underlying mechanisms in human immunology are poorly understood. Pretreatment of murine macrophage cells with very low doses of LPS results in an augmented cytokine production after subsequent LPS stimulation, which is LPS concentration-dependent [40-42]. In general it should be noted that humans are much more sensitive to LPS than mice, indicating the relative poor feasibility of murine models to support human endotoxin responses [43]. The fact that, dependent on the *in vivo* LPS dose applied and specific cytokine measured, either LPS hyporesponsiveness or LPS priming is observed in a relatively narrow LPS dose range (0.5-2 ng/kg) indicates that a delicate balance exists between endotoxin hyporesponsiveness and endotoxin priming, which is still to be characterized in more detail.

It should be noted that sample collection tubes used for *ex vivo* LPS challenges contained an endotoxin-like contamination. Although the exact level of contamination could not be expressed in relative endotoxin units, additional experiments indicated that the contamination was TLR4-specific. As a consequence of this contamination, *ex vivo* LPS challenges were performed at an endotoxin level resulting in a maximal TLR4-mediated response (EC_{100}) rather than the anticipated sub-maximal response level (EC_{80}), which was believed not to affect study outcomes.

Overall, our experiments demonstrate that human endotoxemia induced by commonly applied relatively high LPS doses (exceeding 2 ng/kg) can be avoided: application of LPS doses as low as 0.5ng/kg result in significant responses in routine safety markers (e.g. temperature, blood pressure and heart rate) and circulating cytokine levels that can function as pharmacodynamic markers. As such, the low-dose LPS challenge has been demonstrated to be a feasible methodological tool for future clinical studies exploring pharmacological or nutritional immune-modulating effects. An *in vivo* LPS challenge induced immune cell hyporesponsiveness or immune cell priming (dependent on *in vivo* LPS dose and cytokine readout), determined by repeated *ex vivo* LPS challenges, but the duration of these effects was limited. These results indicate that a combination of *in vivo* LPS administration and repeated *ex vivo* LPS challenges can be applied in clinical pharmacology studies.

Table 1. Contrasts of ANCOVA (difference in %) at 6 hours post-dose after ex vivo LPS challenge.

	0.5 ng/kg vs placebo		1 ng/kg vs placebo		2 ng/kg vs placebo	
	Contrast	P-value	Contrast	P-value	Contrast	P-value
TNFα	-13.6	0.6256	-66.4	0.0005	-74.7	<0.0001
IL1β	-30.0	0.1606	-65.8	<0.0001	-84.7	<0.0001
IL6	34.8	0.0754	-31.3	0.0283	-41.3	0.0024
IL8	55.1	0.0879	19.2	0.4961	-4.8	0.8475

Figure 1. Vital signs: temperature (°C, panel A), heart rate (bpm, panel B), systolic blood pressure (mmHg, panel C), diastolic blood pressure (mmHg, panel D), change from baseline with standard deviation as error bars.

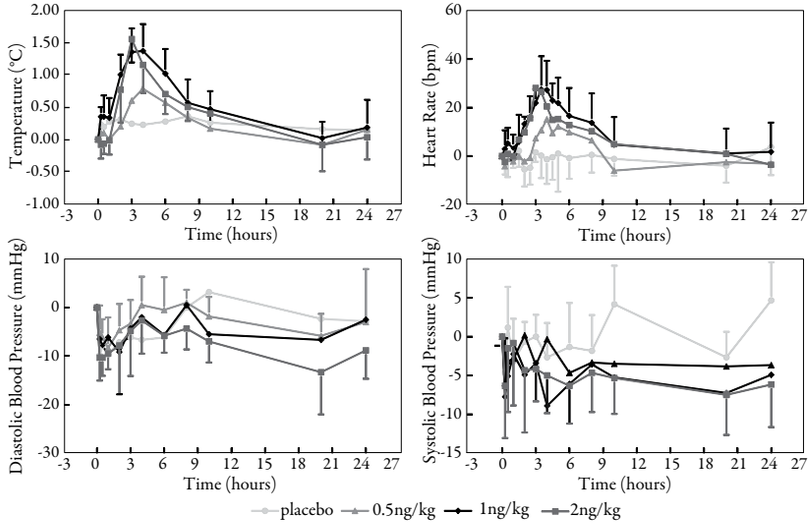


Figure 2. Activated Partial Thromboplastin Time (APTT) LSMs change from baseline profile, with 95% CI as error bars.

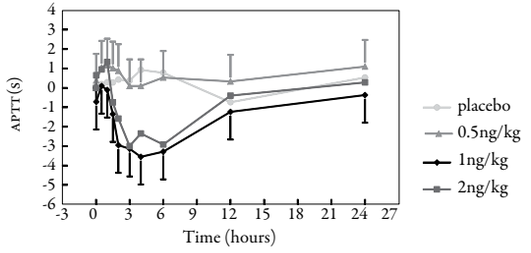


Figure 3. CRP (A) time profile graph, with standard deviation as error bars; TNFα (B), IL6 (C), IL8 (D) time profile graphs up to 6 hours, with standard deviation as error bars.

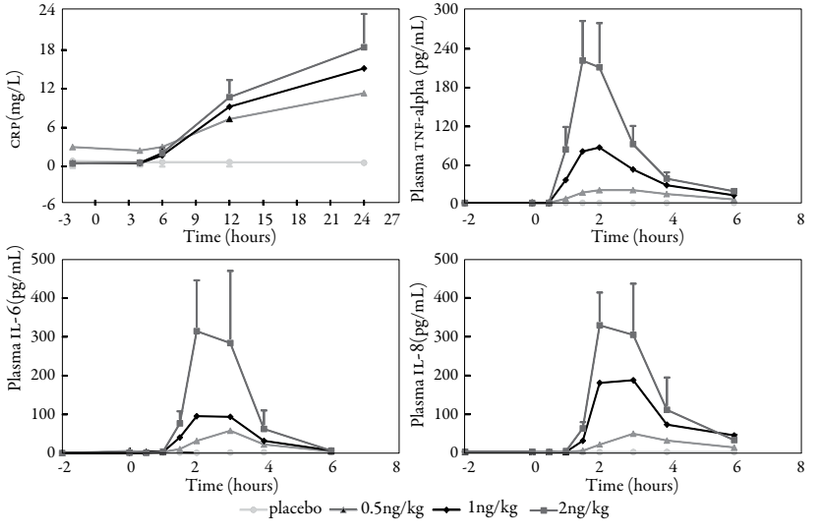
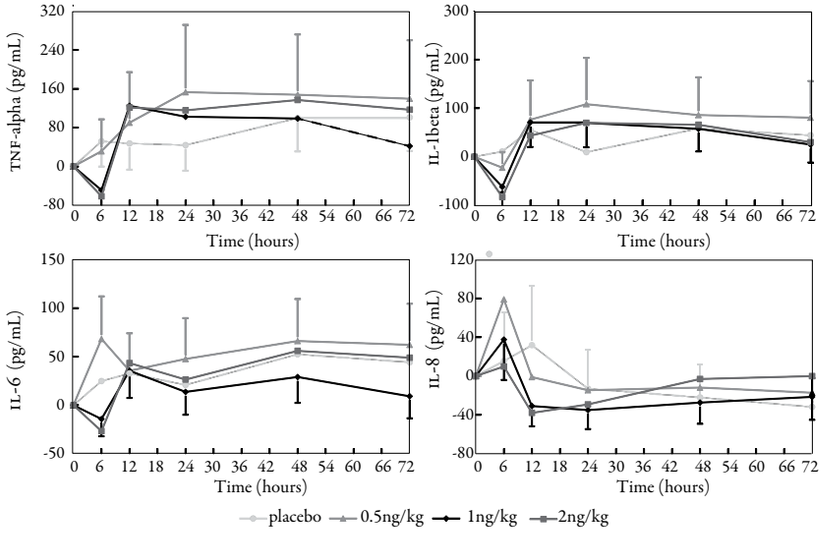


Figure 4. TNFα (A), IL1β (B), IL6 (C), IL8 (D) after ex vivo LPS challenge (10 eu/ng, 24 hour incubation) LSMs change from baseline profile, with 95% CI as error bars, in vivo LPS challenge on t = 0.



REFERENCES

1 Andreasen, A. S., K. S. Krabbe, R. Krogh-Madsen, S. Taudorf, B. K. Pedersen, and K. Moller. 2008. Human endotoxemia as a model of systemic inflammation. *Curr Med Chem.* 15: 1697-1705.

2 Fu, Y., T. Glaros, M. Zhu, P. Wang, Z. Wu, J. J. Tyson, L. Li, and J. Xing. 2012. Network topologies and dynamics leading to endotoxin tolerance and priming in innate immune cells. *PLoS Comput. Biol* 8: e1002526.

3 Fujihara, M., M. Muroi, K. Tanamoto, T. Suzuki, H. Azuma, and H. Ikeda. 2003. Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. *Pharmacol Ther* 100: 171-194.

4 Kox, M., K. S. de, J. C. Pompe, B. P. ramakers, M. G. Netea, J. G. van der Hoeven, C. W. HoedemAEkers, and P. Pickkers. 2011. Differential *ex vivo* and *in vivo* endotoxin tolerance kinetics following human endotoxemia. *Crit. Care Med* 39: 1866-1870.

5 Draisma, A., P. Pickkers, M. P. Bouw, and J. G. van der Hoeven. 2009. Development of endotoxin tolerance in humans *in vivo*. *Crit. Care Med* 37: 1261-1267.

6 van der Poll, T., S. M. Coyle, L. L. Moldawer, and S. F. Lowry. 1996. Changes in endotoxin-induced cytokine production by whole blood after *in vivo* exposure of normal humans to endotoxin. *J Infect. Dis* 174: 1356-1360.

7 de Vos, A. F., J. M. Pater, P. S. van den Pangaart, M. D. de Kruif, '. van, V, and T. van der Poll. 2009. In vivo lipopolysaccharide exposure of human blood leukocytes induces cross-tolerance to multiple TLR ligands. *J Immunol.* 183: 533-542.

8 van Eijk, L. T., R. W. van der Pluijm, B. P. ramakers, M. J. Dorresteijn, J. G. van der Hoeven, M. Kox, and P. Pickkers. 2013. Body mass index is not associated with cytokine induction during experimental human endotoxemia. *Innate. Immun.* 20: 61-67.

9 Ferguson, J. F., C. K. Mulvey, P. N. Patel, R. Y. Shah, J. Doveikis, W. Zhang, J. Tabita-Martinez, K. Terembula, M. Eiden, A. Koulman, J. L. Griffin, N. N. Mehta, R. Shah, K. J. Propert, W. L. Song, and M. P. Reilly. 2013. Omega-3 PUFA supplementation and the response to evoked endotoxemia in healthy volunteers. *Mol Nutr Food Res* 58: 601-613.

10 Nieuwdorp, M., M. C. Meuwese, H. L. Mooij, M. H. van Lieshout, A. Hayden, M. Levi, J. C. Meijers, C. Ince, J. J. Kastelein, H. Vink, and E. S. Stroes. 2009. Tumor necrosis factor-alpha inhibition protects against endotoxin-induced endothelial glycocalyx perturbation. *Atherosclerosis.* 202: 296-303.

11 Draisma, A., G. M. de, C. W. Wouters, N. P. Riksen, W. J. Oyen, G. A. Rongen, O. C. Boerman, D. M. van, J. G. van der Hoeven, and P. Pickkers. 2009. Endotoxin tolerance does not limit mild ischemia-reperfusion injury in humans *in vivo*. *Innate. Immun.* 15: 360-367.

12 Morris, M., and L. Li. 2012. Molecular mechanisms and pathological consequences of endotoxin tolerance and priming. *Arch Immunol. Ther Exp (Warsz.)* 60: 13-18.

13 Kobayashi, K., L. D. Hernandez, J. E. Galan, C. A. Janeway, Jr., R. Medzhitov, and R. A. Flavell. 2002. IraK-M is a negative regulator of Toll-like receptor signaling. *Cell* 110: 191-202.

14 Sly, L. M., M. J. rauh, J. Kalesnikoff, C. H. Song, and G. Krystal. 2004. LPS-induced upregulation of SHIP is essential for endotoxin tolerance. *Immunity* 21: 227-239.

15 Chang, J., S. L. Kunkel, and C. H. Chang. 2009. Negative regulation of MYD88-dependent signaling by IL10 in dendritic cells. *Proc Natl Acad Sci U S A* 106: 18327-18332.

16 Brint, E. K., D. Xu, H. Liu, A. Dunne, A. N. McKenzie, L. A. O'Neill, and F. Y. Liew. 2004. ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. *Nat Immunol.* 5: 373-379.

17 Nakagawa, R., T. Naka, H. Tsutsui, M. Fujimoto, A. Kimura, T. Abe, E. Seki, S. Sato, O. Takeuchi, K. Takeda, S. akira, K. Yamanishi, I. Kawase, K. Nakanishi, and T. Kishimoto. 2002. SOCS-1 participates in negative regulation of LPS responses. *Immunity* 17: 677-687.

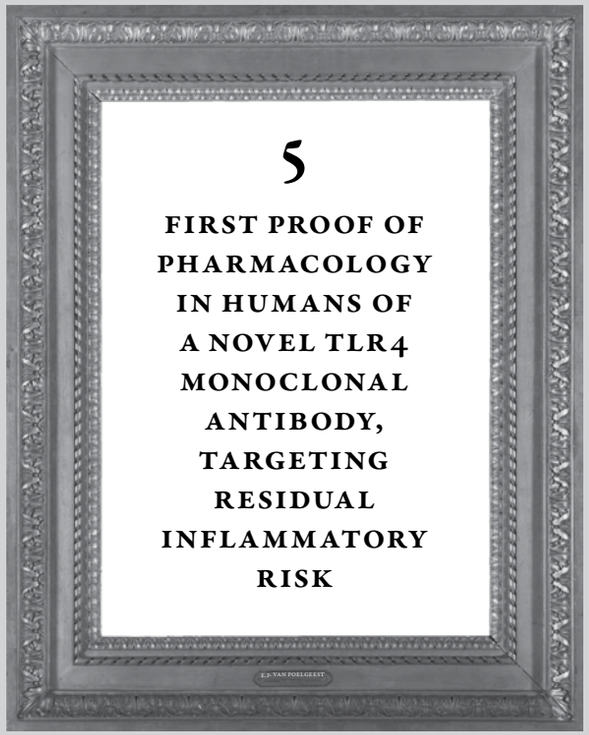
18 Kinjyo, I., T. HanAda, K. InaGaki-Ohara, H. Mori, D. aki, M. Ohishi, H. Yoshida, M. Kubo, and A. Yoshimura. 2002. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. *Immunity* 17: 583-591.

19 Schaaf, B., K. Luitjens, T. Goldmann, B. T. van, F. Sayk, C. Dodt, K. Dalhoff, and D. Droemann. 2009. Mortality in human sepsis is associated with downregulation of Toll-like receptor 2 and CD14 expression on blood monocytes. *Diagn Pathol* 4: 12.

20 van 't Veer, C., P. S. van den Pangaart, M. A. van Zoelen, K. M. de, R. S. Birjmohun, E. S. Stroes, A. F. de Vos, and T. van der Poll. 2007. Induction of IraK-M is associated with lipopolysaccharide tolerance in a human endotoxemia model. *J Immunol.* 179: 7110-7120.

21 SivapalaraTNam, S., R. Farrugia, M. Nieuwdorp, C. F. Langford, R. T. van Beem, S. Maiwald, J. J. Zwaginga, A. Gusnanto, N. A. Watkins, M. D. Trip, and W. H. Ouwehand. 2011. Identification of candidate genes linking systemic inflammation to atherosclerosis; results of a human *in vivo* LPS infusion study. *BMC Med Genomics* 4: 64.

22 Ungerstedt, J. S., A. Soop, A. Sollevi, and M. Blomback. 2003. Bedside monitoring of coagulation activation after challenging healthy volunteers with intravenous endotoxin. *Thromb Res* 111: 329-334.
23 de, J. E., P. E. Dekkers, A. A. Creasey, C. E. Hack, S. K. Paulson, A. Karim, J. Kesecioglu, M. Levi, S. J. van Deventer, and P. T. van Der. 2000. Tissue factor pathway inhibitor dose-dependently inhibits coagulation activation without influencing the fibrinolytic and cytokine response during human endotoxemia. *Blood* 95: 1124-1129.
24 Levi, M., T. van der Poll, and H. R. Buller. 2004. Bidirectional relation between inflammation and coagulation. *circulation* 109: 2698-2704.
25 Esmon, C. T. 2005. The interactions between inflammation and coagulation. *Br J Haematol.* 131: 417-430.
26 Levi, M., T. van der Poll, and C. H. Ten. 2006. Tissue factor in infection and severe inflammation. *Semin Thromb Hemost* 32: 33-39.
27 Franco, R. F., J. E. de, P. E. Dekkers, J. J. Timmerman, C. A. Spek, S. J. van Deventer, D. P. van, K. L. van, G. B. van, C. H. Ten, T. van der Poll, and P. H. Reitsma. 2000. The *in vivo* kinetics of tissue factor messenger RNA expression during human endotoxemia: relationship with activation of coagulation. *Blood* 96: 554-559.
28 Stouthard, J. M., M. Levi, C. E. Hack, C. H. Veenhof, H. A. Romijn, H. P. Sauerwein, and T. van der Poll. 1996. Interleukin-6 stimulates coagulation, not fibrinolysis, in humans. *Thromb HAEmost.* 76: 738-742.
29 van der Poll, T., H. R. Buller, C. H. Ten, C. H. Wortel, K. A. Bauer, S. J. van Deventer, C. E. Hack, H. P. Sauerwein, R. D. Rosenberg, and J. W. ten Cate. 1990. Activation of coagulation after administration of tumor necrosis factor to normal subjects. *N Engl J Med* 322: 1622-1627.
30 van Deventer, S. J., H. R. Buller, J. W. ten Cate, L. A. Aarden, C. E. Hack, and A. Sturk. 1990. Experimental endotoxemia in humans: analysis of cytokine release and coagulation, fibrinolytic, and complement pathways. *Blood* 76: 2520-2526.
31 van Eijk, L. T., M. J. Dorresteijn, P. Smits, J. G. van der Hoeven, M. G. Netea, and P. Pickkers. 2007. Gender differences in the innate immune response and vascular reactivity following the administration of endotoxin to human volunteers. *Crit. Care Med* 35: 1464-1469.
32 Coyle, S. M., S. E. Calvano, and S. F. Lowry. 2006. Gender influences *in vivo* human responses to endotoxin. *Shock* 26: 538-543.
33 Michie, H. R., K. R. Manogue, D. R. Spriggs, A. Revhaug, S. O'Dwyer, C. A. Dinarello, A. Cerami, S. M. Wolff, and D. W. Wilmore. 1988. Detection of circulating tumor necrosis factor after endotoxin administration. *N Engl J Med* 318: 1481-1486.
34 Van Zee, K. J., S. M. Coyle, S. E. Calvano, H. S. Oldenburg, D. M. Stiles, J. Pribble, M. Catalano, L. L. Moldawer, and S. F. Lowry. 1995. Influence of IL1 receptor blockade on the human response to endotoxemia. *J Immunol.* 154: 1499-1507.
35 Dorresteijn, M. J., L. T. van Eijk, M. G. Netea, P. Smits, J. G. van der Hoeven, and P. Pickkers. 2005. Iso-osmolar prehydration shifts the cytokine response towards a more anti-inflammatory balance in human endotoxemia. *J Endotoxin. Res* 11: 287-293.
36 Blackwell, T. S., and J. W. Christman. 1996. Sepsis and cytokines: current status. *Br J AnAEsth.* 77: 110-117.
37 Netea, M. G., C. A. Nold-Petry, M. F. Nold, L. A. Joosten, B. Opitz, J. H. van der Meer, F. L. van de Veerdonk, G. Ferwerda, B. Heinhuis, I. Devesa, C. J. Funk, R. J. Mason, B. J. Kullberg, A. Rubartelli, J. W. van der Meer, and C. A. Dinarello. 2009. Differential requirement for the activation of the inflammasome for processing and release of IL1beta in monocytes and macrophages. *Blood* 113: 2324-2335.
38 Martinon, F., K. Burns, and J. Tschopp. 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL1beta. *Mol Cell* 10: 417-426.
39 Dorresteijn, M. J., A. Draisma, J. G. van der Hoeven, and P. Pickkers. 2010. Lipopolysaccharide-stimulated whole blood cytokine production does not predict the inflammatory response in human endotoxemia. *Innate. Immun.* 16: 248-253.
40 West, M. A., and A. Koons. 2008. Endotoxin tolerance in sepsis: concentration-dependent augmentation or inhibition of LPS-stimulated macrophage TNF secretion by LPS pretreatment. *J Trauma* 65: 893-898.
41 Hirohashi, N., and D. C. Morrison. 1996. Low-dose lipopolysaccharide (LPS) pretreatment of mouse macrophages modulates LPS-dependent interleukin-6 production *in vitro*. *Infect. Immun.* 64: 1011-1015.
42 Deng, H., U. Maitra, M. Morris, and L. Li. 2013. Molecular mechanism responsible for the priming of macrophage activation. *J Biol Chem.* 288: 3897-3906.
43 Munford, R. S. 2010. Murine responses to endotoxin: another dirty little secret? *J Infect. Dis* 201: 175-177.



5

FIRST PROOF OF PHARMACOLOGY IN HUMANS OF A NOVEL TLR4 MONOCLONAL ANTIBODY, TARGETING RESIDUAL INFLAMMATORY RISK

E Monnet, G Lapeyre*, EP van Poelgeest*, P Jacqmin, K de Graaf, J Reijers, M Moerland, J Burggraaf, C de Min. *Evidence of NI-0101 pharmacological activity, an anti-TLR4 antibody, in a randomized phase 1 dose escalation study in healthy volunteers receiving LPS.* Clinical Pharmacology and Therapeutics 2017 Feb;101(2):200-208. (* Equal contribution)

ABSTRACT

Toll-like receptor (TLR) 4 pathways are major contributors to pathological inflammatory responses induced by tissue damage. NI-0101 is the first monoclonal antibody blocking TLR4 signaling. This activity is independent of the ligand type and concentration, therefore blocking potentially any TLR4 ligands. A Phase 1 single ascending dose study was conducted in 73 healthy volunteers (HV) to evaluate NI-0101 tolerability, preliminary safety, pharmacokinetics and pharmacodynamics, in absence and in presence of a systemic challenge with lipopolysaccharide (LPS), a TLR4 ligand. NI-0101 was well tolerated without safety concern. The pharmacokinetic profile was characterized by a half-life of approximately 10 days at high concentrations and by a rapid elimination at low concentrations due to expected target mediated drug disposition. NI-0101 prevented cytokine release following *ex vivo* and *in vivo* LPS administration and also prevented the CRP increase and the occurrence of flu-like symptoms expected following the *in vivo* administration of LPS.

INTRODUCTION

NI-0101 is a humanized IgG1κ monoclonal antibody (mAb) engineered by Novimmune to bind to and block the activation and signaling of human TLR4 by interfering with its dimerization, independently of the ligand type and concentration. This characteristic confers NI-0101 its potential to block any TLR4 ligands present in pathological conditions and inducing inflammatory signals. NI-0101 binds with high affinity to human TLR4 (Kd = 139 pM). NI-0101 engineering destroyed the binding sites for interactions with FcγRIII and the complement component, C1q, while maintaining the binding to FcγRI and to FcγRIIa. This removed the ability of NI-0101 to provoke antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). TLR4 blockade is enhanced by NI-0101 binding to Fcγ receptors [1]. *In vitro* data with human whole blood have shown that a polymorphism at amino acid position 131 (R/H) of the FcγRIIa affects the potency of NI-0101 for blocking TLR4-mediated cytokine release (NI-0101 IC_{50} in LPS-induced IL6 release were 2191, 223 and 128 pM for 131HH, 131HR group and 131RR, respectively).

In vitro and *in vivo* preclinical pharmacology and toxicology studies performed with NI-0101 or a functionally equivalent anti-mouse TLR4 mAb, 5E3, serving as a surrogate molecule, showed no safety concerns (data on file). NI-0101 is being developed for the treatment of inflammatory disease, in particular Rheumatoid Arthritis (RA). Despite advances in the treatment of RA brought by biologic treatments, approximately 40% of patients require switching biological treatments because of inadequate response or loss of response, and only 30% achieved sustained remission [2].

RA is an immunologically driven condition characterized by persistent joint (synovitis) and systemic inflammation, and presence of autoantibodies, in particular against citrullinated proteins [3]. citrullinated proteins and anti-citrullinated protein antibodies (ACPA) can form immune complexes in inflamed joints. These immune complexes are considered to be inflammatory mediators and similarly to the so called Damage-Associated Molecular Pattern molecules (DAMPs) [4, 5, 6]. DAMPs are recognized by TLR family expressed in particular on monocytes and macrophages responsible for innate inflammatory response, and by osteoclasts and chondrocytes contributing to cartilage erosion and destabilizing repair mechanisms [7]. TLR is a family of at least 11 proteins that recognize structurally conserved molecules derived from microbes. The bacterial component, LPS, binds TLR4 [4]. In addition, literature reports that immune complexes formed between citrullinated proteins and their associated ACPA can engage TLR4 and Fcγ

receptors on immune cells [6, 8]. Engagement of immune complexes present in RA patients with FcγRIIa has been shown to enhance inflammation[9] and higher levels of FcγRIIb is prognostic of lower disease activity in RA and dampens TLR4 activation.[10] TLR4 activation leads to cytokine production including TNFα and IL6, which are established therapeutic targets in ra.[11] Investigations using RA human cells stimulated with RA synovial fluids have shown that cytokine release is inhibited by NI-0101 when specific ACPA are present in the RA synovial fluids (data submitted for publication). In addition, 5E3 in the mouse Collagen Induced Arthritis model and the IL1Rn-/- arthritis mouse model prevented disease progression. Based on these data, specific blockade of TLR4 activation by NI-0101 appears as a promising therapeutic approach in RA.

We report the results of the Phase 1 study assessing the initial tolerability, safety, and PK/PD profiles of NI-0101 after infusion of single escalating iv doses in HV. The study aimed to establish evidence of human pharmacology by evaluating the capacity of NI-0101 to prevent the effects of the *ex vivo* and *in vivo* administration of a TLR4 ligand, LPS. In Part 1, NI-0101 was administered at single ascending doses with *ex vivo* LPS challenges. In Part 2, the administration of NI-0101 was followed by both *ex vivo* and *in vivo* LPS challenges. NI-0101 PD profile was evaluated by measuring the levels of IL6 and TNFα (MYD88 pathway) and the levels of IFNγ and CXCL10 (TRIF pathway) after the LPS challenges. *In vivo* LPS effects on CRP, hematological parameters and vital signs were assessed in the presence and absence of NI-0101 to further demonstrate its pharmacological effects. Finally, the anticipated impact of the FcγRIIa polymorphism on NI-0101 PD profile was assessed in the 3 FcγRIIa genotypes HH, RH and RR equally represented in the study.

METHODS

All study documentations were submitted to the Medical Ethics Committee of Academic Medical Center (AMC) in Amsterdam and the CCMO (Central Committee on Research involving Human Subjects; Competent Authority) for approval. The study was conducted in accordance with the principles set forth in the Declaration of Helsinki, the Guidelines of the International Conference on Harmonisation (ICH) on Good Clinical Practice (GCP) (CPMP/ICH/135/95), European Union (EU) Directive 95/46/EC, the Dutch Act on Medical Research involving Human Subjects (Wet Mensgebonden Onderzoek, WMO), and other applicable regulatory requirements.

STUDY DESIGN The study was a randomized, double-blind, placebo-controlled, PK/PD guided, Phase 1 study in HV adults. Subjects were administered for 2 hours iv infusion of either placebo (saline solution) or NI-0101 in saline solution. Part 1 objectives were to assess the tolerability, safety, PK and PD with *ex vivo* LPS challenge after single ascending dose of NI-0101, taking into account the FcγRIIa genotype. Fifty-seven subjects received escalating doses of NI-0101, from 0.001 mg/kg to 15 mg/kg, or placebo. Male subjects were included in all cohorts of both parts. Because of potential effect of menstrual cycle on TLR expression, female subjects were only included from the moment that a certain NI-0101 dose had achieved complete inhibition of *ex vivo* LPS-induced cytokine release in men; female subjects participated in cohorts 3 to 7 of Part 1. Each cohort included at least 2 representatives of each of the 3 FcγRIIa-131 genotypes (H/H, R/R, R/H) who received NI-0101. Genotype was not specified for placebo subjects. Nine subjects were enrolled in the first cohort (NI-0101:placebo, 6:3), and all subsequent cohorts enrolled 8 subjects (NI-0101:placebo, 7:1, including one subject receiving the same dose as the previous cohort). NI-0101 PD effects were assessed by whole blood *ex vivo* stimulation with LPS.

Part 2 objectives were to assess the tolerability, safety, PK and PD with *ex vivo* and *in vivo* LPS challenge after single selected doses of NI-0101. The first cohort (0.01 mg/kg) enrolled 4 subjects (NI-0101:placebo, 3:1) and the second cohort (0.25 mg/kg) enrolled 12 subjects divided into three subgroups (2a, 2b and 2c), each composed of 4 subjects (NI-0101:placebo, 3:1). Subjects also received an *in vivo* LPS challenge at 2 ng/kg, either immediately at the end of NI-0101/placebo infusion (cohort 1 and cohort 2a), or at a delayed time-point (22 or 40 days, for cohort 2b and 2c, respectively) after infusion. For all groups, the selected genotype of the subjects who received active treatment was 131R/R and 131R/H (ratio of 1:2 per group). The genotype of placebo subjects was not specified.

The assignment of subjects and treatments to the randomization schedule was done with SAS (SAS Institute, USA) by CHDR's statistician. All personnel involved in the execution and evaluation of the study (except statistician generating the study randomization schedule) was blinded.

NI-0101 drug product (manufactured by Nova Laboratories Ltd, UK) and matching placebo compositions were L-Histidine (1.88 mg/mL), L-Histidine monohydrochloride monohydrate (2.70 mg/mL), Sucrose (68.46 mg/mL), Polysorbate 80 (0.05 mg/mL), water for injection to 1 mL, pH 6.0. LPS lot#3 was manufactured in the United States of America by the National Institute of Health.

The first subject was enrolled on 29 October 2012 and last subject completed the study on 27 March 2014. All patients were followed until NI-0101 concentration could not be measured or close to the limit of quantification. The study was registered on the following databases: http://www.clinicaltrials.gov (NCT01808469) and on http://eudract.emea.eu.int (2012-003657-28).

DOSE SELECTION As NI-0101 does not bind to TLR4 in any animal species commonly used for preclinical safety assessment, a mouse surrogate antibody (5E3) was used for *in vivo* pharmacology, pharmacokinetics and toxicology studies. In a 4-week toxicity study in mice, no unintended effects of 5E3 were observed at the highest dose tested (240 mg/kg/week intravenously). The Minimal Anticipated Biological Effect Level (MABEL) was assessed by a modeling and simulation approach, using data from *in vitro* human whole blood LPS challenges and target occupancy data, and predicted PK based on other NovImmune antibodies and canakinumab. As consequence, the starting dose for Part 1 of the study was calculated to be 0.001 mg/kg, predicted to result in a 3-37% reduction in *ex vivo* LPS-induced cytokine release, dependent on FcγRIIa genotype. Dose selection for subsequent cohorts was guided by PK/PD data from preceding cohorts.

For Part 2, the starting dose was selected based on *ex vivo* cytokine release inhibition observed in Part 1, with an anticipated inhibition of *in vivo* LPS-induced cytokine production of at least 75% with duration of 3 days. The second dose to be administered was intended to induce prolonged (i.e. up to 28 days) and marked (≥90%) inhibition of *in vivo* LPS-induced cytokine production.

STATISTICAL METHODS All statistical analyses were exploratory; therefore, no formal sample size calculation was performed. As standardly conducted in phase 1 studies to allow initial PK/PD assessment, a minimum of 6 subjects per dose were included in part 1 (a minimum of 2 subjects with each FcγRIIa genotype) and 4 subjects per group were included in part 2 (RH or RR FcγRIIa genotypes only) in order to explore the *in vivo* LPS effect in presence of NI-0101 over time. No adjustments were made to compensate for multiple testing. Placebo subjects from each cohort were pooled into a single placebo group. Safety and PD data were pooled by defined analysis periods in Part 2: outside of the LPS challenge period and 72h post the LPS challenge. For all continuous variables, summary statistics included n, mean, median, standard deviation, minimum and maximum. No descriptive statistics were calculated in the event of very few observations ($n < 3$); if N=2, only the arithmetic mean was calculated; if N=1, the single value was reported. PK parameters were determined using serum concentrations with WinNonlin (Certara, USA) and SAS for dose proportionality assessment, and presented with 3 significant digits, except for T_{max}, which was presented to one decimal place. Change and percentage change from baseline was computed for selected parameters.

FCΓRIIA GENOTYPING DNA was extracted from whole blood using the QiaAmp DNA Blood Mini Kit (Qiagen) and analysis was performed at Eurofins Medigenomix GmbH (Ebersberg, Germany). 50 ng of DNA was used for PCR and sequencing using primers detecting the FcγRIIa-131 R/H mutation.

PK ASSAY The serum concentrations of NI-0101 were determined by Covance Laboratories (Harrogate, UK) using an ELISA on the Gyrolab™ xP immunoassay platform (Gyros, Sweden), using biotin labelled mouse IgG1 kappa anti-NI-0101 idiotype monoclonal antibody 4C4 (Novimmune SA) as capture reagent and Alexa Fluor® 647 labelled 4C4 as detection reagent.

EX VIVO LPS CHALLENGE Six mL of blood were collected in sodium heparin (Greiner). Blood was diluted (1:1) with RMPI 1640 medium with 25 mM Hepes and L-Glutamine in the presence of LPS (2.5 ng/mL). The samples were incubated for 24 hours at 37°C (5% CO_2), then were centrifuged (20 minutes 2000G at 20°C) prior to measure cytokines in the supernatant. IL6, TNFα and CXCL10 were measured with R&D Quantikine® ELISA and Multi-array Meso Scale Discovery® by Good Biomarker Sciences (Leiden, Netherlands). IFNγ was measured by ELISA Verikine HStm (PBL-Interferon source) by Novimmune (Plan-Les-Ouates, Switzerland).

ANTI-DRUG ANTIBODY (ADA) ASSAY Evaluation of Ada against NI-0101 was performed by Covance (Horrogate, UK) using bridging ELISA on the Gyrolab™ xP immunoassay platform, with biotin labelled NI-0101 as capture reagent and Alexa Fluor® 647 labelled NI-0101 as detection reagent, and followed the tiered approach design.

RESULTS

DEMOGRAPHICS Demographic data are summarized in Table 1a and Table 1b for the *ex vivo* (Part 1) and *in vivo* (Part 2) LPS challenges, respectively. Fifty seven subjects (9 received placebo and 48 received NI-0101) were recruited in Part 1 and 16 subjects in Part 2 (4 received placebo and 12 received NI-0101). No differences between cohorts were observed for ethnicity (primarily white), age (mean between 21.0 and 30.3 years) and BMI (mean between 21 and 25 kg/m^2).

SAFETY Overall, single infusions of NI-0101 were well tolerated and no safety concern was identified up to the highest dose of 15 mg/kg. In total 74% of the 73 HV reported 140 treatment-emergent adverse events (TEAEs), including 27 events reported by 8 subjects who received placebo. Overall, the type, intensity, duration,

and frequency of AEs were comparable across the placebo and all NI-0101 groups, as well as across all 3 FcγRIIa genotypes (data not shown). The majority of AEs were mild and resolved within hours to days. None of the AEs was severe or serious and there were no AE-related withdrawals. The most commonly reported AE was headache (35% in NI-0101-treated subjects, and 23% in placebo-treated subjects). The most frequently reported system organ class was infections and infestations with predominantly nasopharyngitis (21 events reported by 20 subjects, time of onset 2-62 days after NI-0101/placebo infusion; most being of mild intensity and not requiring treatment) and gastro-enteritis (3 events reported by subjects at 2 different doses and in placebo groups). Two Gram-negative bacterial infections (*E.coli* urinary tract infections) were reported in 2 female subjects with reported history of urinary tract infections, one following placebo and one following NI-0101 infusion. Nine events attributable to 'Influenza-like illness' were observed in different NI-0101 dose groups; none corresponding to an infusion-related reaction, as their onset were between 12 and 47 days post-infusion. Most of the other AEs occurred as single events across different system organ classes in individual subjects (see Table 2) and are events commonly reported in Phase 1 studies.

Low anti-NI-0101 antibody positive titers were observed post-infusion in 5 out of 60 subjects who received NI-0101 and only at a single time point. The presence of pre-infusion positive titers in 2 of these subjects, in one placebo and the low titers suggest a degree of non-specificity of the assay or cross-reactivity to pre-existing antibodies.

No clinically relevant changes were observed in clinical chemistry, hematology, coagulation tests, vital signs and physical examinations. No trend was noticed in electrocardiogram results and in particular there was no evidence of an effect on the QT_c interval. In few subjects receiving NI-0101 or placebo, sporadic mild elevations of IL6 were observed, as expected as part of the normal variability [12, 13] and without concomitant clinical effects. No elevation was observed for other cytokines following NI-0101 administration. Importantly, these data suggest the absence of TLR4 agonist activity of NI-0101.

PHARMACOKINETICS As shown in Figure 1, NI-0101 maximal mean serum concentrations were achieved between the end of infusion and 2h after end of infusion with the highest value achieved at the dose of 15 ng/mL (367,000 ng/mL) and comparable concentrations between Part 1 and Part 2 (118 and 99.5 ng/mL at 0.01 mg/kg and 5,850 and 5,270 ng/mL at 0.25 mg/kg for Part 1 and 2, respectively). As expected, at the dose of 0.001 mg/kg, NI-0101 serum concentrations were not quantifiable.

The higher the administered dose, the longer the NI-0101 concentrations were quantifiable in serum, i.e. 4 days after infusion of 0.01 mg/kg and up to 18 weeks after infusion of 15 mg/kg (the last sampling time), but close to the limit of quantification. The PK profile of NI-0101 was neither influenced by the administration of LPS nor by the different FcγRIIa genotypes (see supplementary Table S1 online).

The maximal NI-0101 concentration (C_{max}), the end-of-infusion NI-0101 concentration (CE01) and the exposure (AUC_{inf}) increased with the dose (see Table 3). However, a nonlinear profile was observed, particularly at lower doses, because of the expected target mediated drug disposition (TMDD). The PK profiles and the calculation of the clearance per dose group indicated that the terminal elimination of NI-0101 decreased with increasing dose to reach a plateau at the dose of 1 mg/kg. Different phases in the elimination of NI-0101 could be observed, especially at higher doses when the target-mediated elimination pathway was saturated. The half-life of the linear elimination phase, calculated based on the pooled observations between day 20 and 80 of the 15 mg/kg dose group, was estimated to be approximately 10 days (k_{el} = -0.0027 h-1; data not shown). The volume of distribution (V_{ss}) was larger than the total blood volume suggesting a distribution of NI-0101 in the tissues.

PHARMACODYNAMICS *Ex vivo* LPS challenge. The PD profile of NI-0101 obtained after *ex vivo* LPS challenges is presented in Figure 2 for IL6 per FcγRIIa genotype.

A dose-dependent inhibition of IL6, TNFα, CXCL10 and IFNβ (see supplementary Table S2 online) release induced by an *ex vivo* LPS challenge was observed after a single administration of NI-0101 compared to placebo. The inhibitory effect was comparable for all cytokines measured, lasted longer when the dose of NI-0101 was increased and was influenced by the FcγRIIa genotype. For the FcγRIIa genotype H/H (Figure 2A), inhibition of IL6 release was 49.0% at the 0.05 mg/kg dose of NI-0101 and > 90% at the dose of 0.25 mg/kg and at higher doses. For the FcγRIIa genotype R/H (Figure 2B) and R/R (Figure 2C), the average inhibition of IL6 release at the end of the NI-0101 infusion was already > 70% and > 80%, respectively, at the 0.01 mg/kg dose of NI-0101 and > 90% at the dose of 0.05 mg/kg and at higher doses. The average duration of a significant inhibition (>80%) of cytokine release increased with the increase of the dose to reach between 8 weeks and 14 weeks at the 15 mg/kg dose, depending on the FcγRIIa genotype (H/H<<R/H<R/R).

IN VIVO LPS CHALLENGE NI-0101 pharmacological effects were studied in Part 2 of the study by the administration of an *in vivo* LPS challenge at three different

time-points after the end of NI-0101 infusion in three separated subgroups of HV (end of NI-0101 infusion, 22 days later and 40 days later).

In subjects administered placebo, an increase in serum IL6 concentration was observed with a maximal measured concentration achieved at 2h after the LPS challenge (Figure 3A). In subjects administered NI-0101 at the 0.01 or 0.25 mg/kg dose and who received the LPS challenge immediately after the infusion, no increase in serum cytokine concentration was observed. This absence of IL6 induction was also observed in subjects exposed to the LPS challenge 22 days after NI-0101 infusion. However, in subjects administered NI-0101 at a dose of 0.25 mg/kg and exposed to the LPS challenge 40 days after the NI-0101 infusion, an increase in serum IL6 concentration was observed with a maximal measured concentration achieved post LPS challenge approximately after 2h. Similar increase in serum concentration was also observed for TNFα, CXCL10 and IFNγ (data not shown).
Consistent with the observation on cytokines, LPS induced CRP release was prevented by NI-0101 up to 22 days after iv infusion at a dose of 0.25 mg/kg (Figure 3B). Similarly, prevention of LPS induced changes in neutrophils and leucocytes counts, coagulation factor (aPTT), heart rate (data not shown) and temperature (Figure 3C) was observed in presence of NI-0101.

Overall, duration of NI-0101 effect after *in vivo* and *ex vivo* LPS administration was comparable.

DISCUSSION

We report on the first-in-human administration of NI-0101, an antibody binding and blocking TLR4, while ENGAGING FcγRI and FcγRII. In this study, single ascending doses of NI-0101 were administered to HV. *Ex vivo* and *in vivo* LPS challenges allowed assessing the pharmacological activity of NI-0101. Dose escalation decisions and follow-up duration were guided by interim analysis and modeling of PK/PD data.

Similarly to previous clinical experiences with other molecules blocking TLR4[14,15], as well as with an anti-TLR2 antibody16, NI-0101 was well-tolerated in HV at doses up to 15 mg/kg with no apparent dose-event or genotype-event relationship. No infusion-related adverse events were reported. No clinically relevant increases of cytokines were observed. The absence of indications for increased susceptibility to gram negative bacteria for the duration of TLR4 blockade (up to 14 weeks following the administration of NI-0101 at 15 mg/kg) is in line with published data on TLR4-independent responses to LPS, such as autophagy, endocytosis, phagocytosis, oxidative burst and inflammasome activation[17,18] demonstrating that the immune system is endowed with alternative pathways for a single offensive stimulus.

The observed PK profile of NI-0101 was non-linear due to the expected TMDD at low NI-0101 concentrations and was characterized by a long half-life of approximately 10 days as reported by most mAb-targeting cell surface receptors.[16,19,20,21]The elimination of NI-0101 decreased with increasing doses, reaching a plateau at 1 mg/kg, likely reflecting the expected saturation of the targeted receptor, as previously described for an antibody targeting TLR2.[16] The presence of a TLR4 ligand (i.e. LPS) *in vivo* did not influence the PK of NI-0101. Considering this observation and the known ligand independent effect of NI-0101 *in vitro*, it can be speculated that the presence of TLR4 ligands in pathological conditions will not influence NI-0101 PK. Finally, NI-0101 elimination was not affected by the FcγRIIa-131 R/H polymorphism.

Consistent with NI-0101 PK, MYD88 (CXCL10 and IFNγ) and TRIF (IL6 and TNFα pathways were completely blocked in blood upon the administration of NI-0101 from the dose of 1 mg/kg onwards. At lower doses, NI-0101 achieved incomplete blockade of cytokine release induced by LPS. The maximum duration of complete inhibition also increased with the dose, up to 14 weeks for TNFα (supplementary Table S3) at the highest dose tested (15 mg/kg) depending on the genotype. The long lasting effect observed for the higher doses of NI-0101 is associated with the well-known long half-life of monoclonal antibodies. This PK/PD characteristic gave the opportunity to perform an initial safety evaluation of the consequences of a prolonged inhibition of the TLR4 pathway, up to 14 weeks. It also offers flexibility in the choice of dosing regimen which can be adapted to satisfy the requirements to study NI-0101 at different development stages or in different indications.

As predicted by the PK/PD simulations, subjects with an FcγRIIa HH genotype showed a shorter duration of inhibition compared to RR or RH genotypes. This observation was consistent with previous IC_{50} data reported from *in vitro* experiments (internal report), which was considered in the design of the study where FcγRIIa genotype (HH) was selected to be enrolled in the sentinel group of the first cohort as a safety precaution. Future studies will address the effect of the FcγRIIa genotype on clinical response.
In Part 2 of the study, the *in vivo* effects of LPS on clinical parameters (such as body temperature and heart rate) and laboratory parameters (CRP, aPTT, platelets and leucocytes) were completely prevented by the administration of NI-0101. These *in vivo* observations in humans provide a confirmation of the hypothesized mechanism of action of NI-0101.

The magnitude and duration of the effects of NI-0101 were well comparable after *ex vivo* and *in vivo* LPS challenges, allowing using *ex vivo* data to predict *in vivo* pharmacological effects.

Taken together, the PK/PD data generated in HV in the presence and absence of a TLR4 ligand can be used to generate a robust PK/PD model for the prediction of the PK/PD profile in patients, therefore likely reducing the risk for the selection of the expected effective dose. Based on the data from this study and assumptions specific to the disease of interest (target tissue, chronic versus acute disease…), expected NI-0101 pharmacological effects can be simulated in relation to its concentration, providing a sound rational to select the dose and frequency of administrations for Phase 2 studies.

In conclusion, this study has demonstrated NI-0101 good tolerability, a favorable safety and PK profile and a robust and durable anti-inflammatory effect in HV. The *ex vivo* LPS challenge appeared to be a reliable surrogate for the *in vivo* pharmacodynamics profile of NI-0101. The prevention of *in vivo* LPS effects provides evidence of NI-0101 pharmacological activity. These data enable the further development of NI-0101 in a wide range of diseases that are mediated through the activation of TLR4 and provide flexibility in the choice of dosing regimen. In particular, NI-0101 appears a promising treatment to block efficiently major inflammatory mediators signaling through TLR4 which are present in the synovial fluid and blood of RA patients.

Table 1A. Baseline characteristics Part 1.

		Placebo	NI-0101						
			0.001	0.01	0.05	0.25	1	5	15
		N=9	N=7	N=7	N=7	N=7	N=7	N=7	N=6
FcγRIIa	H/H	4	3	2	2	2	2	2	2
	R/H	5	2	3	3	3	3	3	2
	R/R	0	2	2	2	2	2	2	2
Sex	Female	2	0	0	1	1	1	1	2
	Male	7	7	7	6	6	6	6	4
Age	Mean	26.8	23.4	22.6	24.7	21.1	24.1	24.0	21.0
	SD	7.84	5.38	2.15	5.41	1.86	5.93	3.51	0.00
BMI	Mean	23.58	21.16	21.39	23.41	21.46	24.99	22.44	23.72
	SD	3.063	1.045	1.419	3.456	1.760	3.248	1.69	33.276

Abbreviations: BMI: body mass index; H/H, H/R, R/R: FcγRIIa-131 polymorphisms.

Table 1B. Baseline characteristics Part 2.

		Placebo	NI-0101				
			0.01 mg/kg		0.25 mg/kg		All
			Immediate*	Immediate*	Day 22*	Day 40*	
		(N=4)	(N=3)	(N=3)	(N=3)	(N=3)	(N=9)
FcγRIIa	H/H	2	0	0	0	0	0
	R/H	2	2	2	2	2	6
	R/R	0	1	1	1	1	3
Sex	Female	0	0	0	0	0	0
	Male	4	3	3	3	3	9
Age	Mean	27.5	24.0	25.0	30.3	22.3	25.9
	SD	3.11	4.00	4.36	8.62	3.51	6.23
BMI	Mean	22.45	21.37	23.57	23.47	23.17	23.40
	SD	2.740	1.266	1.762	4.539	4.203	3.221

Abbreviations: BMI: body mass index; H/H, H/R, R/R: FcγRIIa-131 polymorphism; SD: standard deviation.

** Immediate: lipopolysaccharide administration immediately after NI-0101 administration (study day 1), at study day 22 or 40, respectively.*

Table 2. Adverse events reported in at least 2 subjects by preferred term by treatment group (excluding AEs reported during 72h after in vivo endotoxin administration).

	Placebo	NI-0101							All
		0.001	0.01	0.05	0.25	1	5	15	
	(N=13)	(N=7)	(N=10)	(N=7)	(N=16)	(N=7)	(N=7)	(N=6)	(N=73)
	N (E)	N (E)	N (E)	N (E)	N (E)	N (E)	N (E)	N (E)	N (E)
Headache	3 (3)	1 (1)	1 (1)	3 (5)	5 (7)	2 (4)	4 (4)	5 (5)	**24 (30)**
Nasopharyngitis	4 (5)	2 (2)	2 (2)	2 (2)	0	2 (2)	4 (4)	4 (4)	**20 (21)**
Influenza like illness	0	0	2 (2)	0	1 (1)	2 (2)	2 (2)	2 (2)	**9 (9)**
Myalgia	2 (2)	0	2 (2)	0	2 (3)	0	0	1 (1)	**7 (8)**
Dizziness	0	0	3 (3)	0	0	1 (1)	0	0	**4 (4)**
Oropharyngeal pain	0	0	3 (3)	1 (1)	0	0	0	0	**4 (4)**
Nausea	2 (2)	0	0	1 (1)	1 (1)	0	0	0	**4 (4)**
Gastroenteritis	1 (1)	0	0	0	1 (1)	0	0	1 (1)	**3 (3)**
Catheter site hematoma	1 (1)	0	0	0	0	0	0	1 (1)	**2 (2)**
Back pain	0	2 (2)	0	0	0	0	0	0	**2 (2)**
Musculoskeletal chest pain	0	0	1 (1)	0	0	0	1 (1)	0	**2 (2)**
Cough	1 (1)	0	1 (1)	0	0	0	0	0	**2 (2)**
Rhinorrhoea	0	0	1 (1)	0	1 (1)	0	0	0	**2 (2)**
Feeling hot	0	0	0	0	1 (1)	0	1 (1)	0	**2 (2)**
Bacteriuria/UTI	1 (1)	0	0	0	0	1 (1)	0	0	**2 (2)**
Catheter site pain	2 (2)	0	0	0	0	0	0	0	**2 (2)**

N= number of subjects
E= number of events

Table 3. Summary of NI-0101 Pharmacokinetic Parameters and Dose Proportionality Assessment

	Part1						Part 2		Dose
	NI-0101dose								prop*
	0.01 (N=7)	0.05 (N=7)	0.25 (N=7)	1 (N=7)	5 (N=7)	15 (N=6)	0.01 (N=3)	0.25 (N=9)	Rdnm#
C_{max} (ng/mL)	118 (24.2)	918 (18.0)	5850 (12.6)	30.6*103 (31.4)	118*103 (11.4)	367*103 (15.5)	99.5 (16.2)	5270 (16.9)	1.93 (1.56-2.4)
T_{max} (h)	2.0 (2.0-2.0)	4.0 (2.0-6.0)	2.2 (2.0-6.0)	4.0 (2.0-6.1)	4.0 (4.0-6.1)	3.0 (2.0-4.1)	2.1 (2.0-2.1)	2.1 (2.0-4.2)	NA
AUC_{inf} (h.ng/mL)	3490 (49.2)	67.7*103 (28.0)	809*103 (12.3)	559*103 (25.4)	241*105 (30.3)	811*105 (23.3)	2740 (29.1)	745*103 (16.5)	4.8 (9.5-22.9)
$t_{1/2}$ (h)	21.9 (21.3)	44.5 (10.4)	73.8 (22.5)	104 (19.1)	131 (20.7)	132 (31.0)	21.5 (15.7)	70.7 (27.7)	NA
CL (L/h)	0.251 (45.0)	0.0593 (23.7)	0.022 (10.6)	0.0161 (25.7)	0.0162 (25.5)	0.0146 (22.2)	0.288 (30.1)	0.0260 (18.9)	NA
V_{ss} (L)	6.87 (32.5)	4.06 (18.6)	3.11 (11.4)	3.62 (17.0)	4.55 (9.21)	4.71 (18.7)	8.12 (22.1)	3.83 (16.8)	NA

N: number of subjects in specified group; NA: not applicable; C_{max}: maximum serum concentration; t_{max}: time to maximum serum concentration; AUC_{inf}: area under the concentration-time curve; $t_{1/2}$: half-life; CL: Clearance; Vss: volume of distribution at steady state.
Note: PK parameters for NI-0101 dose group 0.001 mg/kg are not presented, as all except 1 concentration were not quantifiable.
Values are arithmetic mean (CV%) except median (min-max) for t_{max}.
* Dose proportionality for Part 1 assessed by the Power Model Statistical analysis.
# Rdnm: model-predicted geometric means ratio for high and low dose, for dose normalized PK parameters.

Supplementary table 1. Mean NI-0101 serum concentrations (ng/mL) per genotype

	0.01 mg/kg			0.1 mg/kg			0.25 mg/kg			1 mg/kg			5 mg/kg			15 mg/kg		
	H/H	R/H	R/R	H/H	R/H	R/R	H/H	R/H	R/R	H/H	R/H	R/R	H/H	R/H	R/R	H/H	R/H	R/R
2 hrs	115	121	115	939	919	809	5320	5970	5810	24100	30100	24900	108000	123000	96300	389000	384000	306000
4 hrs	94.4	110	101	900	943	782	5400	5650	5740	24500	35000	25900	105000	120000	112000	365000	384000	320000
6hrs	87.4	102	96.3	754	932	756	5280	5460	5790	24100	36500	24400	105000	125000	105000	357000	353000	310000
12 hrs	68.1	80.7	65.3	701	868	723	5060	5140	4440	22800	25700	17500	88800	110000	95000	333000	352000	297000
1 day	46.3	54.7	41.1	483	680	573	3930	4480	4210	17400	24800	20700	81500	95500	83400	300000	310000	219000
2 days	22.8	28.8	17	424	537	428	3390	3750	3700	19600	20400	17700	70400	80400	69800	251000	273000	211000
3 days	12.1	13.6	6.64	276	403	296	2640	2950	3110	14200	17300	15800	62100	65300	52500	206000	232000	164000
4 days	5.59	6.77	0	218	283	235	2390	2500	2760	14200	15900	11600	51300	56800	47800	174000	201000	144000
1 wk	0	0	0	60.4	133	84.5	1690	1700	2070	8320	14400	9180	37600	41900	34300	122000	135000	119000
2 wks	6.15	0	0	0	4.97	0	531	584	883	5350	5850	5040	19600	25700	19700	73700	87900	70200
3 wks	0	0	0	0	0	0	143	132	315	3210	3490	2850	10700	17000	12500	43000	54300	43500
4 wks	0	0	0	0	0	0	16.3	12	68	1480	1890	1520	6600	12400	7320	29200	37500	28100
6 wks	0	0	0	0	0	0	0	0	0	187	434	321	2320	5230	2600	11000	15700	11400
8 wks	0	0	0	0	0	0	0	0	0	4.33	39.2	15.7	625	2300	653	4720	7820	5930
10 wks													125	1020	58.6	1820	3050	2470
12 wks													8.92	515	0	780	1220	387
14 wks																319	405	153
16 wks																72.3	67.4	NC
18 wks																8.11	8.62	16.9

NC : Not calculated (N=1).

Supplementary table 2. Percentage of inhibition from baseline of ex vivo LPS-induced cytokine release, per genotype, for cohorts receiving 1 and 15 mg/kg NI-0101.

	TNFα							CXCL10							IFNβ					
	Placebo	1 mg/kg			15 mg/kg			1 mg/kg			15 mg/kg			1 mg/kg			15 mg/kg		
		H/H	R/H	R/R	H/H	R/H	R/R	H/H	R/H	R/R	H/H	R/H	R/R	H/H	R/H	R/R	H/H	R/H	R/R
2hrs	4.72	**-100**	**-100**	**-99.7**	**-100**	**-100**	**-100**	**-93.5**	**-98.5**	**-94.7**	-71.6	**-100**	**-100**	**-100**	**-100**	**-100**	**-100**	**-100**	**-100**
12hrs	36.9	**-100**	**-100**	**-100**	**-99.8**	**-100**	**-100**	**-94.1**	**-97.9**	**-97.3**	-78.9	**-95.2**	**-95.1**	**-100**	**-100**	**-100**	**-100**	**-100**	**-100**
24hrs	-17.7	**-100**	**-100**	**-100**	**-100**	**-100**	**-100**	**-94.4**	-100	**-97.8**	-79.7	**-100**	**-100**	**-100**	**-100**	**-100**	**-100**	**-100**	**-100**
2days	-22.3	**-100**	**-100**	**-100**	**-100**	**-100**	**-100**	**-98.1**	-100	**-97.8**	**-94.3**	**-100**	**-100**	**-100**	**-100**	**-100**	**-100**	**-98.9**	**-100**
3days	-8.68	**-100**	**-100**	**-100**	**-100**	**-100**	**-100**	**-97.0**	-100	**-97.6**	**-84.3**	**-100**	**-100**	**-100**	**-100**	**-100**	**-99.5**	**-100**	**-100**
7days	-12.9	**-98.8**	**-100**	**-100**	**-100**	**-100**	**-100**	**-92.1**	-100	**-97.8**	**-95.1**	**-100**	**-100**	**-100**	**-100**	**-100**	**-100**	**-100**	**-100**
2wks	-14.2	**-100**	**-100**	**-100**	**-100**	**-100**	**-100**	**-93.9**	-100	**-98.4**	**-89.1**	**-98.8**	**-100**	**-100**	**-100**	**-100**	**-100**	**-100**	**-100**
3wks	9.29	**-91.8**	**-99.0**	**-100**	**-99.8**	**-100**	**-100**	-5.78	**-96.1**	**-97.3**	**-86.6**	**-100**	**-100**	**-92.1**	**-100**	**-100**	**-100**	**-100**	**-100**
4wks	-24.9	**-85.9**	**-98.7**	**-99.7**	**-99.1**	**-100**	**-100**	1.25	**-93.7**	**-97.8**	-78.5	**-100**	**-100**	**-82.2**	**-100**	**-100**	**-100**	**-100**	**-100**
6wks	-21.7	**-6.11**	**-76.9**	**-97.9**	**-100**	**-100**	**-36.7**	17.5	**-68.5**	**-75.6**	-100	**-100**	**-60.9**	-55.4	-82.6	**-95.1**	**-100**	**-100**	**-100**
8wks	-28.1	-48.0	31.8	9.16	**-85.1**	**-100**	**-100**	13.4	175	9.22	8.26	**-100**	**-100**	47.7	32.5	10.4	-58.1	**-100**	**-100**
10wks	-31.9	484			**-48.6**	**-100**	**-99.4**				53.6	**-100**	**-100**				-48.9	**-100**	**-100**
12wks	-49.5				-20.3	**-98.3**	**-86.3**				71.6	**-93.2**	-22.0				-13.3	**-97.7**	**-89.5**
14wks	-25.5				14.9	**-87.4**	**-31.8**				-19.2	**-86.9**	-48.8				-18.6	**-94.6**	-67.8
16wks	-29.3				1.24	-25.1	-4.60				-21.8	-44.4	1.34				-23.8	-59.9	-45.2
18wks	-0.578				36.1	-10.8	148				16.3	-22.6	-8.04				33.0	-27.7	126

In bold: data points with percentage change from baseline ≥ -80%. Values are arithmetic mean. H/H, R/H, R/R: FcγRIIa genotypes; LPS: lipopolysaccharide.

Figure 1. Mean NI-0101 serum concentrations (ng/mL) after single ascending dose of NI-0101 Part 1 (black); Part 2 (grey).

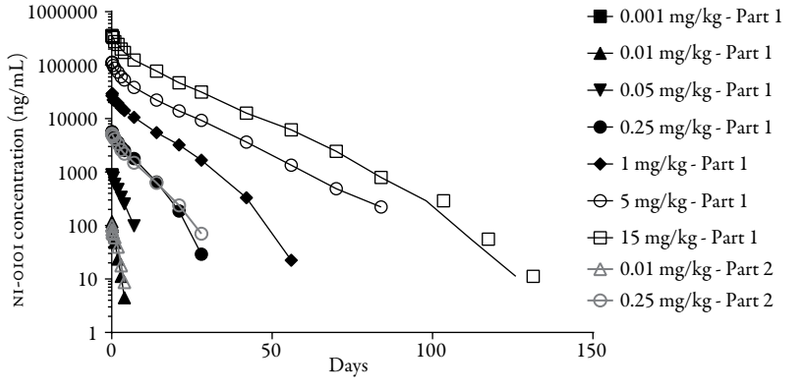


Figure 2. Mean IL6 percentage change from baseline per genotype (*ex vivo* LPS Challenge) in Part 1 FcγRIIa genotype H/H (A), R/H (B), R/R (C).

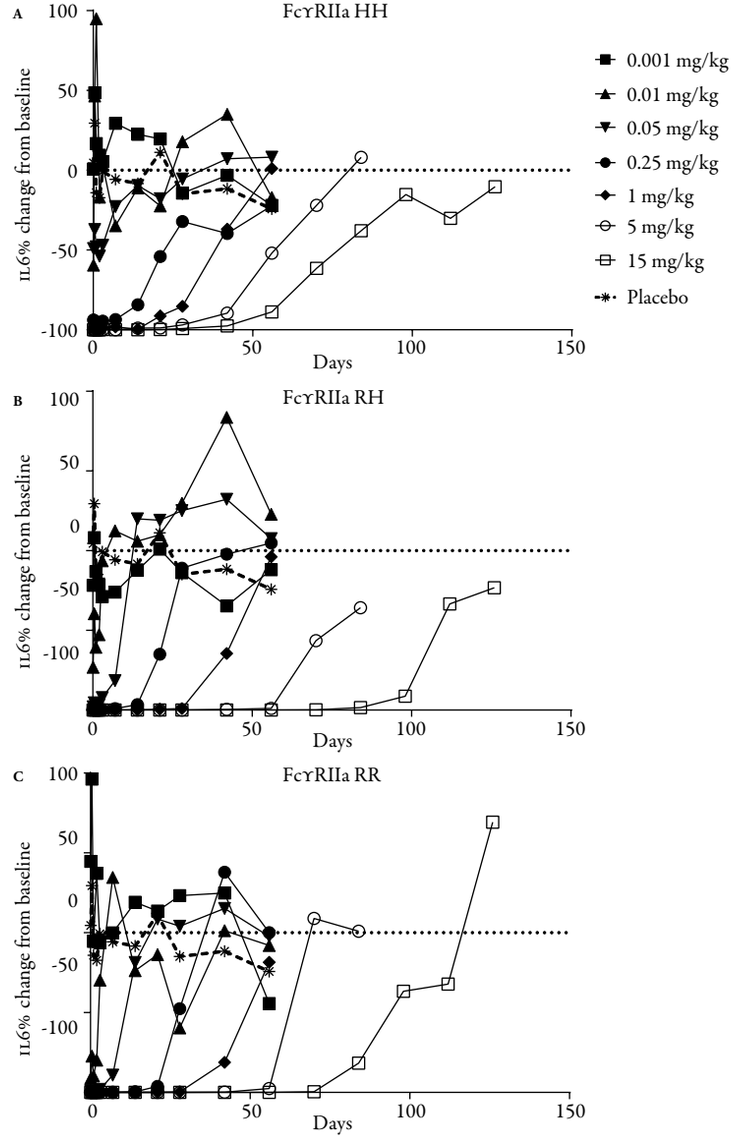
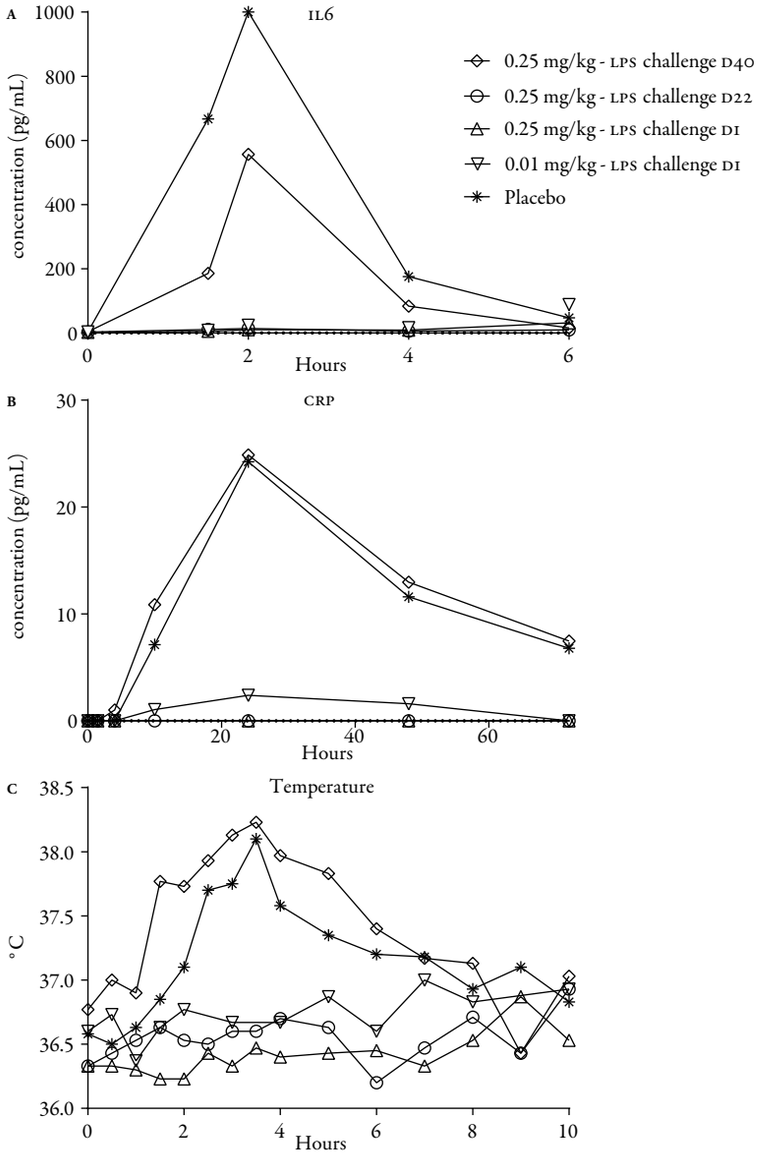
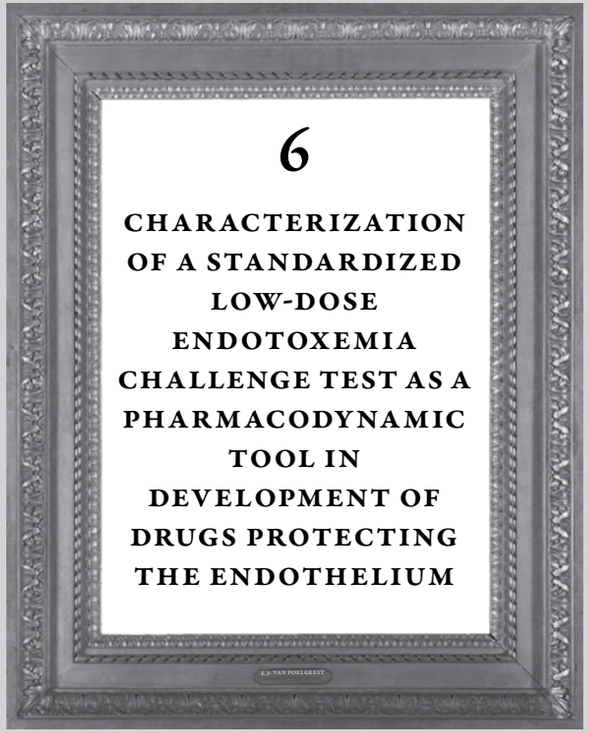


Figure 3. Change in pharmacological parameters after *in vivo* LPS Challenge in Part 2 mean IL6 serum concentration (pg/mL; panel A), (B) mean CRP serum concentration (mg/L; panel B), and temperature (panel C).



REFERENCES

1 Dunn-Seigrist et al. Pivotal involvement of Fcg receptor IIA in the neutralization of lipopolysaccharide signaling via a potent novel anti-TLR4 monoclonal antibody 15C1. JBC. 282(48):34817-34827 (2007)
2 Curtis et al. The use of biologics in Rheumatoid Arthritis: current and emerging paradigms of care. Clinical therapeutics. 33(6):679-707 (2011)
3 Klareskog et al. Rheumatoid Arthritis. Lancet. 373(9664):659-72 (2009)
4 O´Neill et al. Therapeutic targeting of toll-like receptors for infectious and inflammatory diseases and cancer. Pharmacol. Rev. 61:177-197 (2009)
5 Shotorbani et al. Toll-like receptors are potential therapeutic targets in rheumatoid arthritis. World J. Biol. Chem. 2(7):167-72 (2011).
6 Sokolove et al. Immune Complexes Containing citrullinated Fibrinogen Costimulate Macrophages via Toll-like Receptor 4 and FcγReceptor. Arthritis & rheumatism. 63(1):53-62 (2011)
7 Bobacz et al. Toll-like receptors and chondrocytes. Arthritis & rheumatism. 56(6):1880-93 (2007)
8 Zhao et al. circulating immune complexes contain citrullinated fibrinogen in rheumatoid arthritis Arthritis Research & Therapy. 10:R94 (2008)
9 Clavel et al. Induction of macrophage secretion of tumor necrosis factor alpha through Fcgamma receptor IIA engagement by rheumatoid arthritis-specific autoantibodies to citrullinated proteins complexed with fibrinogen. Arthritis Rheum. 58(3):678-88 (2008)
10 Wenink et al. The inhibitory Fc gamma IIb receptor DAMPens TLR4-mediated immune responses and is selectively up-regulated on dendritic cells from rheumatoid arthritis patients with quiescent disease. J Immunol. 183(7):4509-20 (2009)
11 Govoni et al. Therapeutic options after treatment failure in rheumatoid arthritis or Spondyloarthritides. Adv. Ther. 31:780-802 (2014)
12 Haack et al. Diurnal variations of interleukin-6 plasma levels are confounded by blood drawing procedures. Psychoneuroendocrinology. 27:921-31 (2002)
13 Vgontzas et al. IL6 and its circadian secretion in humans. Neuroimmunomodulation. 12:131-40 (2005)
14 Rice et al. A randomized, double-blind, placebo-controlled trial of TAK-242 for the treatment of severe sepsis. Crit Care Med. 38: 1685-1694 (2010)
15 Barochia et al. Eritoran tetrasodium (E5564) treatment for sepsis: review of preclinical and clinical studies. Expert. Opin. Drug Metab Toxicol. 7: 479-494 (2011)
16 Reilly M, et al. randomized, double-blind, placebo-controlled, dose-escalating phase I, healthy subjects study of intravenous OPN-305, a humanized anti-TLR2 antibody. Clin. Pharmacol. Ther. 94(5):593-600 (2013).
17 Hagar et al. Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. Science. 341(6151):1250-3 (2013).
18 Kayagaki et al. Noncanonical inflammasome activation by intracellular LPS independent of TLR4. Science. 341(6151):1246-9 (2013).
19 Burmester GR, et al. Mavrilimumab, a human monoclonal antibody targeting GM-CSF receptor-alpha, in subjects with rheumatoid arthritis: a randomised, double-blind, placebo-controlled, phase I, first-in-human study. Ann. Rheum. Dis. 70(9):1542-9 (2011).
20 Hale G, et al. Pharmacokinetics and antibody responses to the CD3 antibody otelixizumab used in the treatment of type 1 diabetes. J. Clin. Pharmacol. 50(11):1238-48 (2010).
21 Yin D, Barker KB, Li R, Meng X, Reich SD, Ricart AD, et al. A randomised phase 1 pharmacokinetic trial comparing the potential biosimilar PF-05280014 with trastuzumab in healthy volunteers (REFLECTIONS B327-01). Br. J. Clin. Pharmacol. (2014).



6

CHARACTERIZATION OF A STANDARDIZED LOW-DOSE ENDOTOXEMIA CHALLENGE TEST AS A PHARMACODYNAMIC TOOL IN DEVELOPMENT OF DRUGS PROTECTING THE ENDOTHELIUM

EP van Poelgeest, MR Dillingh, KE Malone, EM Kemper, ESG Stroes, J Burggraaf, M Moerland. *Characterization of immune cell, endothelial, and renal responses upon human low-dose experimental endotoxemia.* Journal of Pharmacological and Toxicological Methods 2018; 89: 39-46.

ABSTRACT

BACKGROUND Although the effects of relatively high concentrations of endotoxin on endothelial activation/dysfunction and kidney markers has been described in literature, detailed insight in the LPS concentration-effect relationship, the magnitude, variability and timing of the response, and potential effects of endotoxemia on the kidneys is lacking. A study was performed to assess the effects of low- to moderate dose (0.5, 1 or 2 ng/kg) endotoxemia on the endothelium and kidneys as measured by a panel of novel highly sensitive kidney injury markers.

METHODS This was a randomized, double-blind, placebo-controlled study with single ascending doses of LPS (0.5, 1 or 2 ng/kg) administered to healthy male volunteers (3 cohorts of 8 subjects, LPS:placebo 6:2). Endothelial measures included selectins, cell adhesion molecules, and thrombomodulin. Renal measures included novel, sensitive and specific biomarkers of acute kidney injury.

RESULTS Endotoxin exposure resulted in consistent LPS dose-dependent responses in inflammatory markers, E- and P- Selectin, VCAM1, ICAM1, and thrombomodulin. The observed biological responses were transient, reaching a level of significance of at least <0.01 in the highest dose group and with an effect size which was dependent on the administered LPS dose. LPS-induced inflammatory and endothelial effects did not translate into a change in renal damage biomarkers, although at 2 ng/kg LPS, subtle and transient biomarker changes were observed that may relate to (subclinical) tubular damage.

DISCUSSION We demonstrated that administration of a single LPS dose of 2 ng/kg to healthy volunteers results in significant inflammatory and endothelial responses, without inducing clinically relevant signs of kidney injury. These findings support the application of the human endotoxemia model in future clinical pharmacology studies.

INTRODUCTION

The human endotoxemia model is a well-established model for investigation of the physiological mechanisms of systemic inflammation. In this experimental setting, purified lipopolysaccharide (LPS, also referred to as endotoxin) from the cell membrane of *Escherichia Coli* is administered intravenously to healthy volunteers resulting in flu-like signs and symptoms, and increased levels of inflammatory markers (cytokines and acute phase reactants). Recently, we published the results of a clinical study characterizing the inflammatory response at low to moderate LPS doses (0.5, 1, and 2 ng/kg) administered to healthy volunteers, focusing on clinical signs and symptoms and cytokine responses [1]. We demonstrated that this human endotoxemia model is a safe and robust methodological tool, feasible for use in clinical pharmacology studies to assess the anti-inflammatory effect of new investigational compounds.

Whereas the effect of *in vivo* endotoxin exposure on clinical signs and cytokine release in humans has been described extensively [2-4], limited information is available on the effect of lower LPS doses on the human endothelium and kidneys. This is in contrast with the detailed knowledge on the effects of endotoxin at a molecular/cellular level: LPS induces an inflammatory response via stimulation of Toll-like receptors (TLRs), innate immune receptors activated by tissue damage or by molecules associated with pathogen-associated molecular patterns (PAMPs) on invading microorganisms. Subsequent activation of multiple intracellular inflammatory pathways (e.g. MYD88-dependent and TRIF-dependent pathways) results in pro-inflammatory cytokine/chemokine release by leucocytes. LPS-induced cytokine/chemokine release leads to selectin-mediated rolling of leucocytes, firm adhesion of the leucocytes to the endothelium via cellular adhesion molecules and integrins, and transmigration of these cells through the endothelium to fight infection [5,6]. This protective inflammatory response, however, is accompanied by collateral tissue damage through inflammatory factors and reactive oxygen species secreted by cytokine-activated immune cells. The balance between protection and damage inflicting inflammation is delicate, and in various pathological conditions the beneficial effect of the inflammatory response may be outweighed by its harmful effects. In sepsis, for example, systemic inflammation and microvascular dysfunction ultimately leads to acute kidney injury (AKI) [7].

There are reports showing that LPS doses of 2-4 ng/kg activate the endothelium, reflected by an acute increase in circulating P and E-Selectin, vascular cell adhesion molecule (VCAM)1, intercellular adhesion molecule (ICAM)1, and thrombomodulin levels [7,8]. However, systematically collected and quantitative

data providing detailed insight in the relationship between LPS dose and vascular response are lacking. Moreover, data linking endotoxin-induced activation of the human vasculature and (subclinical) kidney injury are not readily available in the public domain. A human *in vivo* model with controlled and transient activation of the endothelium could qualify the human endotoxin challenge as a pharmacodynamic model for the early clinical development of new drugs, and non-pharmacological interventions such diet and life-style. This model would not only be relevant for new therapies targeting immune cells, but potentially also for compounds developed to improve or maintain integrity of the endothelium. Endothelial dysfunction is recognized as a key process in various pathological conditions including atherosclerosis [9-11], diabetes, insulin resistance [12] and sepsis-associated kidney injury [13], so pharmacological protection of the endothelial lining may be an important future target. Since LPS-induced systemic inflammation and endothelial activation may ultimately affect renal function, it is of importance that also the potential effects of the *in vivo* LPS challenge on the human kidney are quantified, to assure that this response is limited and transient.

We investigated the effect of an endotoxin challenge on endothelial activation, renal function and kidney injury markers. Healthy human volunteers were exposed to a single dose of LPS (0.5, 1 or 2 ng/kg, or placebo). The effects on the endothelium and the kidneys were quantified, in relation to the inflammatory response (cytokine release; presented earlier [1]). Endothelial activation measures included selectins, cell adhesion molecules, and TM. Renal measures included routine blood (e.g. creatinine, blood urea nitrogen) and urine (e.g. fractional excretion of sodium, sediments) markers of kidney injury, but also more sensitive and specific biomarkers for early detection of acute kidney injury (e.g. kidney injury molecule 1 (KIM1)) [14-16].

MATERIAL AND METHODS

Study setup The characteristics of our study have been published previously [1]. In short, 24 healthy male volunteers aged 18-28 years with normal kidney function (Modification of Diet in Renal Disease (MDRD) calculated creatinine clearance >60 mL/minute) and no abnormalities in urinary screen participated in this study, which was performed at the Centre For Human Drug Research in Leiden, The Netherlands. Participants were pre-hydrated with 1500 mL 2.5% glucose/saline (2.5% glucose/0.45% sodium chloride) for 2hrs. Subsequently, they received an intravenous single LPS dose (*Escherichia Coli* 113:H, 10:K negative, U.S. Standard Reference Endotoxin lot#3) or placebo (0.9% sodium chloride). The ratio LPS : placebo per dose group was 6 : 2, and doses comprised 0.5, 1 or 2 ng/kg bodyweight. Volunteers and investigators were blinded for LPS/placebo. Subjects were hydrated with 150 mL/hr glucose/saline by intravenous drip for a period of 6hrs after LPS/placebo administration. Blood samples were collected frequently over time at predefined time points. Urine samples were collected pre-dose, and during the 0-4hrs, 4-8hrs, 8-12hrs and 12-24hrs post-dose time intervals. All subjects provided written informed consent prior to study participation. The study was approved by the Medical Ethics Committee of the Amsterdam Medical Center, The Netherlands, and conducted in compliance with Dutch law on experiments in humans.

Bioanalysis Endothelial activation was measured using MesoScale Discovery's vascular injury panel 1 and V-PLEX™ vascular injury panel 2 (ICAM)1, ICAM3, (VCAM)1, TM, E-Selectin and P-Selectin). An enzymatic method was used to measure serum creatinine, and glomerular filtration rate was estimated using the 4-variable MDRD formula [17] and the chronic kidney disease epidemiology (CKD-EPI) equation (assumed to perform better than the MDRD formula in normal kidney function) [18]. Analysis of exploratory kidney injury biomarkers was performed for urinary beta2-microglobulin (B2MG; Immulite® 2000, a solid-phase two-side chemiluminescent immunometric assay), alpha-gluthatione S-transferase (αGST; Argutus Medical Alpha GST EIA enzyme immunoassay), N-acetyl-β-D-Glucosaminidase (NAG; N-acetyl-β-D-Glucosaminidase assay, Diazyme Europe GmbH), and KIM1 (Quantikine® human TIM-1/KIM-1/HAVCR immunoassay), and the MesoScale Discovery human Kidney Injury Panel V, containing Cystatin C, epidermal growth factor (EGF), neutrophil gelatinase-associated lipocalin (NGAL/Lipocalin-2), osteopontin (OPN), and uromodulin (UMOD; placebo and 2 ng/kg LPS groups only). For αGST measurement and the kidney injury panel, 100 µL of stabilizing buffer (BIO85STB, Argutus Medical) was added to 400 µL of urine.

Statistics All parameters are graphically presented as absolute readings per treatment group. Repeatedly measured parameters were analyzed after log-transformation, using a mixed model of variance with treatment, time, and treatment by time as fixed factors, subject as random factor and the baseline measurement as covariate. Treatment effects were calculated for each parameter for the time period from baseline up to 24hrs, except for the circulating cytokines for which the period was restricted to 6hrs post-dose. Contrasts (LPS versus placebo) were reported with the estimated difference, the 95% confidence interval (CI), and the p-value. All statistical analyses were performed using SAS for Windows Version 9.1.3 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Demographics, safety and circulating cytokines Demographic and baseline characteristics showed no significant differences between groups (Table 1). Administration of LPS resulted in a transient inflammatory response, as published by our group previously [1]. In short, LPS administration dose-dependently increased body temperature (+1.5°C for 2 ng/kg LPS) and heart rate (+28 bpm for 2 ng/kg LPS). LPS exposure resulted in elevated levels of C-reactive protein (CRP) and circulating cytokines, with clearly distinctive increases from placebo already at the lowest LPS dose level tested [0.5 ng/kg, contrast for timeframe 0-6hrs: tumor necrosis factor alpha (TNFα), +413%; interleukin 6 (IL6), +288%; and interleukin 8 (IL8), +254%; all p≤0.0001]. Maximal cytokine concentrations were measured in the 2 ng/kg group at 1.5-3hrs after LPS administration (TNFα: 222±68; IL6: 315±130; IL8: 329±84 pg/mL). CRP concentration was still increasing 24hrs after LPS dose administration.

Endothelial activation markers In the placebo-treated subjects (Figure 1A-E), all endothelial activation markers remained relatively stable over time. LPS administration dose-dependently increased endothelial activation markers (Figure 1, Table 2). An immediate increase after LPS dose administration was observed for circulating levels of VCAM1, TM, and P-Selectin. At an LPS dose of 2 ng/kg, for all these markers the responses reached a level of significance of at least <0.01 (Table 2). For ICAM1 and E-Selectin, a lag time of at least 2 hours was observed. The maximal responses in the 2 ng/kg LPS group for TM, E-Selectin and P-Selectin were reached at 6 hours after LPS administration, and amounted 4.1±0.5, 107±38 and 40±6 versus 3.3±0.7,15±3 and 24±4 ng/mL at baseline, respectively. For ICAM1 and VCAM1 the maximal response was only reached at 24 hours after LPS infusion and amounted 551±44 and 665±24, versus 320±57 and 432±29 ng/mL respectively at baseline. The response of ICAM3 to LPS administration was limited, and did not reach a level of statistical significance at any of the LPS dose levels tested.

Renal function and renal activation markers When expressed as urinary mass per hour (Figure 2A), effects of study-related hydration and diurnal effects were observed. In the placebo-treated group, baseline serum creatinine levels after prehydration amounted 78±4 μmol/L. Levels decreased in the course of the morning, to reach a minimum of 69 μmol/L observed at 6 hours after placebo administration (Figure 2B). In the subsequent hours, serum creatinine returned to baseline. In the LPS-treated subjects, serum creatinine levels followed a non-dose dependent pattern. No clear indications for an LPS-dependent effect on serum creatinine levels were observed (Table 2, Figure 2B). In the placebo-treated group, the estimated GFR (eGFR; calculated using the MDRD formula) after prehydration was 109±6 mL/min/1.73m^2 at baseline, increased in the course of the morning and reached a maximum increase to 126 μmol/L at 8 hours after placebo administration (Figure 2C). In the subsequent hours, eGFR returned to baseline. No clear indications for an LPS-dependent effect on eGFR were observed (Table 2, Figure 2C).

Baseline urinary B2MG concentration (Figure 3A, left panel) was 18±7 μg/L in the placebo-treated subjects, which is in line with previous published data (range in healthy volunteers 20 to 83 μg/L 19). Urinary B2MG concentration increased during the day in the placebo-treated subjects. Maximal concentration amounted 39±16 μg/L and was reached at 24 hours. This may be explained by relative hypovolemia after overnight fasting (see methods and discussion sections). Administration of 2 ng/kg LPS resulted in elevated urinary B2MG concentrations in the 8-24hrs time interval after LPS administration. This elevation amounted a maximum of 84±66 μg/L at 12hrs after LPS administration. The estimated difference over the 0-24hrs interval versus placebo was +88%, p=0.003 (Table 2). Correction for urinary creatinine excretion (Figure 3A, right panel) blunted this effect, although the LPS effect remained statistically significant (estimated difference over the 0-24hrs interval versus placebo +31%, p=0.03; Table 2).

Baseline urinary KIM1 and OPN concentrations (Figures 3B and 3C, left panel, respectively) were 0.18±0.07 and 389±288 μg/L, respectively in placebo-treated subjects. Urinary KIM1 and OPN concentrations increased over day in the placebo-treated subjects. Maximal concentrations were reached at 24 hours after placebo administration, amounting 0.58±0.2 and 883±413 μg/L for KIM1 and OPN, respectively. Administration of 2 ng/kg LPS resulted in elevated urinary KIM1 and OPN concentrations in the interval from 8 hours to 24 hours after LPS administration. This elevation amounted a maximum of 1.27±0.8 μg/L at 24 hours for KIM1, and of 1520±941 μg/L for OPN, respectively. The estimated differences over the 0-24hrs interval versus placebo were 121%, p=0.003 for KIM1, and 85%, p=0.04 for OPN (Table 2). Correction for urinary creatinine excretion (Figures 3B and C, right panel) blunted this effect. For KIM1 this corrected LPS effect remained statistically significant (estimated difference over the 0-24hrs interval versus placebo 44%, p=0.006, whereas for OPN, there was no statistically significant effect anymore (estimated difference over the 0-24hrs interval versus placebo 23%, p=0.5; Table 2).

Also for other renal activation markers (NAG, Cystatin C, UMOD, EGF, NGAL; Figures 3D to 3H), an increase in urinary concentration over day was observed

in the placebo-treated subjects. For these markers, no significant effects of LPS treatment could be observed (Table 2). However, for NAG, UMOD and EGF, the estimated contrasts between LPS and placebo (Table 2) and the graphical time courses (Figures 3D, 3F and 3G, respectively) did suggest the possibility of LPS-dependent effects: the estimated differences between 2 ng/kg LPS and placebo amounted 51% (p=0.1), 36% (p=0.08), and 44% (p=0.07) for NAG, UMOD and EGF, respectively (Table 2). For a considerable number of urinary NGAL and αGST measurements (33% and 89% of total, respectively), readings were below the limit of quantification (6 and 6.25 µg/L, respectively). Therefore, statistical analyses are not presented for these parameters. When evaluated on an individual basis, though, we observed that in the placebo and 0.5 and 1 ng/kg LPS dose groups there were almost no measurable αGST concentrations, whereas in the 2 ng/kg LPS group 3 out of 6 individuals showed measurable levels of αGST excretion at 12hrs after LPS administration, ranging from 10.6 to 84.3 ng/mL (range in healthy volunteers 1.1 to 64 ng/mL) [20].

DISCUSSION

LPS administration dose-dependently increased endothelial activation markers. Although it has been described previously that administration of 2 and 4 ng/kg LPS increases the circulating levels of P- and E-Selectin, VCAM1, ICAM1, and TM [7,8], the quantitative and temporal effects of lower LPS doses on these markers are unclear. We provided these insights, and demonstrated that VCAM1, TM, P-Selectin, ICAM1 and E-Selectin are suitable measures for monitoring LPS-induced endothelial activation. P-selectins promote endothelial activation. E-selectins are expressed on activated endothelial cells and mediate the adhesion of immune cells at sites of inflammation. ICAM1 and VCAM1 interact with leucocytes and are involved in subsequent transmigration into the tissues. TM is a cell surface-expressed glycoprotein present on vascular endothelial cells with an important role in coagulation and complement regulation. The explored markers all demonstrated a very low variability over day, with changes from baseline not exceeding 5% in the placebo group. For most parameters the maximal LPS response was observed at 6hrs post-dose, with the exception of VCAM1 which required a longer period (24hrs) to reach its maximal response upon LPS exposure. For ICAM1 and E-Selectin a lag time in LPS response was observed which most likely relates to cytokine-induced gene transcription [20,21]. The response of ICAM3 to LPS administration was limited, which probably relates to the fact that ICAM3 is constitutionally expressed on resting leucocytes but not on endothelium [22], and its expression is not increased by inflammation [23]. Power calculations were performed which showed that for E-Selectin in a parallel study design, at an LPS dose level of 2 ng/kg, a sample size of 3 per treatment group will have a power of 0.82 to detect a 70% inhibition; a sample size of 15 per group will have a power of 0.82 to detect a 30% inhibition using a two-sample t-test with a 2-sided significance level of 0.05. Under the same conditions, it would be possible to demonstrate a 70% and 30% inhibition with a sample size of 6 and 28 per treatment group, respectively, with a power of 84% and 81%, respectively for VCAM1. These power calculations support the application of low to moderate LPS dose-induced endothelial response as methodological tool for monitoring of drug effects in future pharmacology studies, even though our study was based on a relatively small sample size of 6 subjects per treatment arm. This is valuable information, since the pharmacological inhibition of adhesion molecules has been demonstrated so far predominantly in preclinical studies[24-28], and sporadically in human studies with generally high LPS doses (4 ng/kg) [29].

In addition to the effect on endothelial activation markers, we also explored whether exposure to a single low to moderate dose of LPS would translate into an effect on the kidneys, as endothelial alterations are important in the etiology and progression of renal damage [30]. Since experimental endotoxemia may occasionally result in vasovagal collapse [31], we decided to administer glucose/saline to all study participants from 2hrs pre-dose up to 6hrs post-dose. In the placebo-treated group, urinary volume doubled and eGFR increased by 17% within the first 4-8hrs and had normalized within 12hrs post-dose; serum creatinine levels decreased by a maximum of 12% 6hrs post-dose and had also normalized within 12hrs post-dose. Obviously, the glucose/saline infusion comprising of a total volume of 2.4L over 8hrs may have played an important role in these responses. In the LPS treated subjects, no clear indications were observed for LPS-related changes in kidney function, underlining the renal safety when considering the LPS challenge a methodological tool for future pharmacology studies. However, parameters like urinary volume, serum creatinine and GFR are relatively crude measures when assessing potential effects of an intervention on the kidneys. For example, even administration of 4 ng/kg LPS did not change traditional kidney function measurements creatinine and urea clearance, fractional sodium excretion and total 24hr urinary volume in healthy volunteers [32,33]. These traditional kidney function measurements lack sensitivity and fail to detect early subtle signs of kidney injury [34], while the extent of injury and poor outcomes associated with acute kidney injury worsen with delayed recognition of impending damage. We therefore selected an extensive panel of sensitive and early kidney injury markers

to provide insight into the functionality of different anatomical locations of the nephron. Important issues to consider regarding our renal injury marker data are the effects of overnight fasting and (pre-) hydration that may have influenced urinary concentrations of these markers. After overnight fasting there is relative hypovolemia resulting in elevated concentrations of serum creatinine and solutes in the urine due to decreased urinary volume. (Pre-) hydration restores intravascular volume, leading to an increase in glomerular filtration and increased urinary output, and therefore decreased serum creatinine and solutes in the urine. These diurnal and hydration effects were indeed observed for urinary mass (volume), serum creatinine, eGFR, and urinary concentration of most kidney biomarkers in both the placebo and LPS treated groups. However, there appears to be an untoward effect of the 2 ng/kg LPS dose as significant increases in urinary concentrations of B2MG, KIM1 and OPN were observed in the time interval 8-24hrs after LPS administration. B2MG, which is shed from lymphocytes, is filtered freely across the glomerulus and completely reabsorbed by proximal tubular cells [35]. KIM1 is a transmembrane protein expressed by proximal tubular epithelial cells with phagocytic capacity [35], and a sensitive marker for renal injury [36]. OPN is a secreted phosphoprotein with renal expression that is induced in the distal tubule upon inflammation [37,38]. Because of these characteristics, the observed changes in B2MG, KIM1 and OPN may be indicative of subtle tubular damage. Importantly, however, we observed that correction for urinary volume blunted this effect (absolute B2MG, KIM1 and OPN excretion did not differ between treatment groups). For NAG, Cystatin C, UMOD, EGF, no significant effects of LPS treatment could be observed, although for some of these measures the estimated contrasts between LPS and placebo and the graphical time courses did suggest the possibility of LPS-dependent effects. In summary, an LPS dose of 2 ng/kg does not result in transient kidney dysfunction or overt expression of kidney injury markers, but it does induce some injury responses with borderline significance. Possibly, a further increase in LPS exposure may result in more distinct kidney effects. These data are in line with the observation that administration of 4 ng/kg LPS to healthy volunteers did result in significant increases in B2MG excretion over time [33]. In the same study, NAG excretion was enhanced. This is indicative of tubular dysfunction, and clinical data indicate that NAG can be considered an early marker of mild tubular injury [39]. It may also be useful to compare the observed responses in kidney injury markers with the responses reported in literature in case of overt kidney injury. For example, reported urinary B2MG and KIM1 concentrations in drug-induced and septic AKI were ~3 to 4 fold higher than in our study [40,41]. Moreover, urinary KIM1 levels corrected for urinary creatinine levels in patients with AKI from various etiologies (including, but not limited to nephrotoxicity and sepsis) amounted 3-10 ng/mg creatinine [42-44]. In our study, the highest LPS dose increased KIM1 excretion to a maximum of 0.8 ng/mg creatinine (data not shown). These comparisons indicate that the observed responses in our study are only moderate. For αGST it was difficult to draw conclusions on LPS effects due to a considerable number of measurements below the limit of quantification (6.25 μg/L). It is known that in healthy volunteers urinary αGST concentrations are very low, since αGST is a general detoxification enzyme that is produced in situations of cellular stress/inflammation. An increased αGST excretion may reflect toxicity-induced tubular dysfunction/injury [45,46]. This is in line with our findings: αGST was undetectable except for 3 out of 6 individuals in the 2 ng/kg LPS group at 12hrs after LPS administration. An enhanced αGST excretion upon LPS administration (2 ng/kg) has been described before in a group of healthy volunteers [33].

In summary, we demonstrated that administration of a single low to moderate dose of LPS to healthy volunteers is well-tolerated and induces a sufficiently robust inflammatory [1] and metabolic [47] response that is apparently devoid of untoward renal effects. Also, we showed a dose-dependent transient endothelial response (E- and P-Selectin, VCAM1, ICAM1, and TM), reaching a level of significance at a dose level of 1-2 ng/kg. These findings support the application of the human endotoxemia model in future clinical pharmacology studies.

Table 1. Demographic characteristics.

	LPS			Placebo
	0.5 ng/kg (N=6)	1 ng/kg (N=6)	2 ng/kg (N=6)	(N=6)
Age (yrs)	23 (4)	22 (2)	24 (3)	22 (2)
Weight (kg)	72.6 (6.8)	67.1 (10.1)	73.0 (8.5)	74.3 (9.3)
BMI (kg/m2)	22.1 (1.3)	20.7 (1.5)	22.2 (2.2)	21.4 (2.4)

BMI = body mass index; SD = standard deviation; N=number of subjects in treatment group;
*Placebo subjects were pooled for the three different cohorts.

Table 2. Group contrasts. Data are presented as estimated treatment effect (with 95% confidence interval) for 0.5, 1 and 2 ng/kg LPS versus placebo (pooled for the three different cohorts) from baseline up to 24 hours post-dose.

	0.5 ng/kg LPS vs placebo	1 ng/kg LPS vs placebo	2 ng/kg LPS vs placebo
ENDOTHELIAL ACTIVATION MARKERS			
Thrombomodulin	-6 (-15 - 5)% p=0.3	8 (-4 – 21)% p=0.2	16 (5 - 29)% p=0.008
ICAM1	13 (3 – 23)% p=0.01	30 (19 – 42)% p<.0001	42 (30 - 55)% p=<.0001
ICAM3	-10 (-23 - 4)% p=0.1	6 (-9 - 23)% p=0.4	10 (-8 - 32)% p=0.3
VCAM1	8 (-3 – 20)% p=0.1	20 (9 – 32)% p=0.001	25 (13 - 38)% p=0.0001
E-Selectin	35 (2 – 79)% p=0.03	110 (56 – 182)% p<.0001	280 (186 - 404)% p=<.0001
P-Selectin	-5 (-26 – 21)% p=0.7	17 (-10 - 51)% p=0.2	42 (11 - 82)% p=0.009
RENAL FUNCTION			
Urinary mass	-28 (-64 - 47)% p=0.3	-10 (-53 - 72)% p=0.7	-47 (-73 - 3)% p=0.06
Serum creatinine	7 (-0.2 - 16)% p=0.06	-3 (-9 – 4)% p=0.4	6 (-1 -13)% p=0.1
eGFR (MDRD)	-9 (-18 - -0.5) p=0.04	3 (-6 - 11) p=0.5	-7 (-15 - 1.6) p=0.1
KIDNEY INJURY MARKERS			
Urinary B2MG	30 (-12 - 91)% p=0.2	19 (-19 – 74)% p=0.4	88 (27 - 178)% p=0.003
B2MG/Creat	-4.6 (-26 – 23)% p=0.7	20 (-6 – 53)% p=0.12	31 (3-67)% p=0.03
Urinary KIM1	32 (-25 - 132)% p=0.3	9 (-34 – 79)% p=0.7	121 (38 - 256)% P=0.003
KIM1/Creat	12 (-15 - 47)% p=0.4	15 (-10 - 48)% p=0.2	44 (13 – 83)% p=0.006
Urinary NAG	-18 (-54 - 47)% p=0.5	69 (-8 - 210)% p=0.08	51 (-14 - 167)% p=0.1
NAG/Creat	-19 (-49 - 28)% p=0.3	65 (0.9 - 171)% p=0.05	11 (-31 - 80)% p=0.7
Urinary Cystatin C	--	--	20 (-57 – 231)% p=0.6
Cystatin/Creat			0 (-44 - 79)% p=0.99
Urinary OPN	--	--	85 (3 - 235)% p=0.04
OPN/Creat			23 (-39 - 145)% p=0.5
Urinary UMOD	--	--	36 (-4 - 94)% p=0.08
UMOD/Creat			-9.8 (-42 - 40)% p=0.6
Urinary EGF	--	--	44 (-4 – 116)% p=0.07
EGF/Creat			-4 (-37 - 47)% p=0.83

ICAM1 indicates intercellular adhesion molecule 1; ICAM3, intercellular adhesion molecule 3; VCAM1, vascular cell adhesion molecule 1; eGFR, estimated glomerular filtration rate; MDRD, modification of diet in renal disease; B2MG, beta2-microglobulin; KIM1, kidney injury molecule 1; NAG, N-acetyl-β-D-glucosaminidase; OPN, osteopontin; UMOD, uromodulin and EGF, epidermal growth factor.

Figure 1. Serum concentrations ± SD (error bars) of endothelial activation markers: ICAM1 (A), VCAM1 (B), E-Selectin (C), P-Selectin (D), and TM (E). LPS administration was at T=0.
For readability, only the standard deviations of the two extremes are shown.

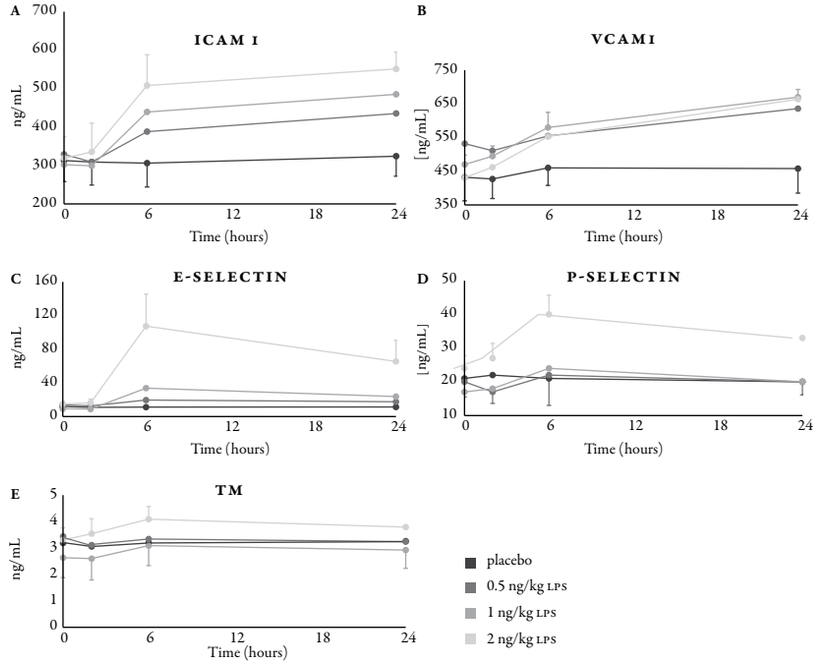


Figure 2. Absolute urinary mass ± SD (error bars) in grams of collected urine excreted per hour (A), and serum concentrations of renal function measurements serum creatinine (µmol/L) (B) and estimated glomerular filtration rate ± SD (error bars) according to the MDRD formula (mL/min/1.73m²) (C). LPS administration was at T=0. For readability, only the standard deviations of the placebo and 2 ng/kg LPS group are shown in panels B and C.

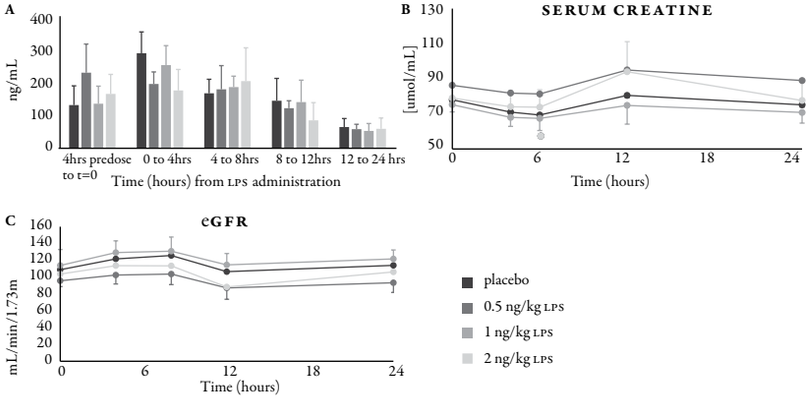
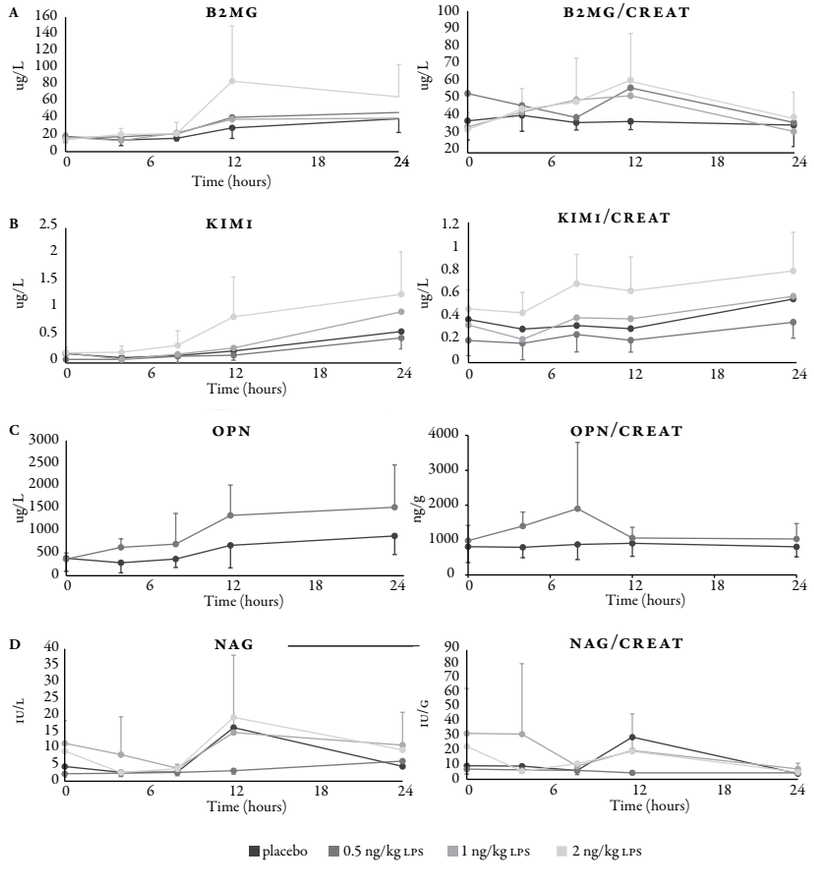
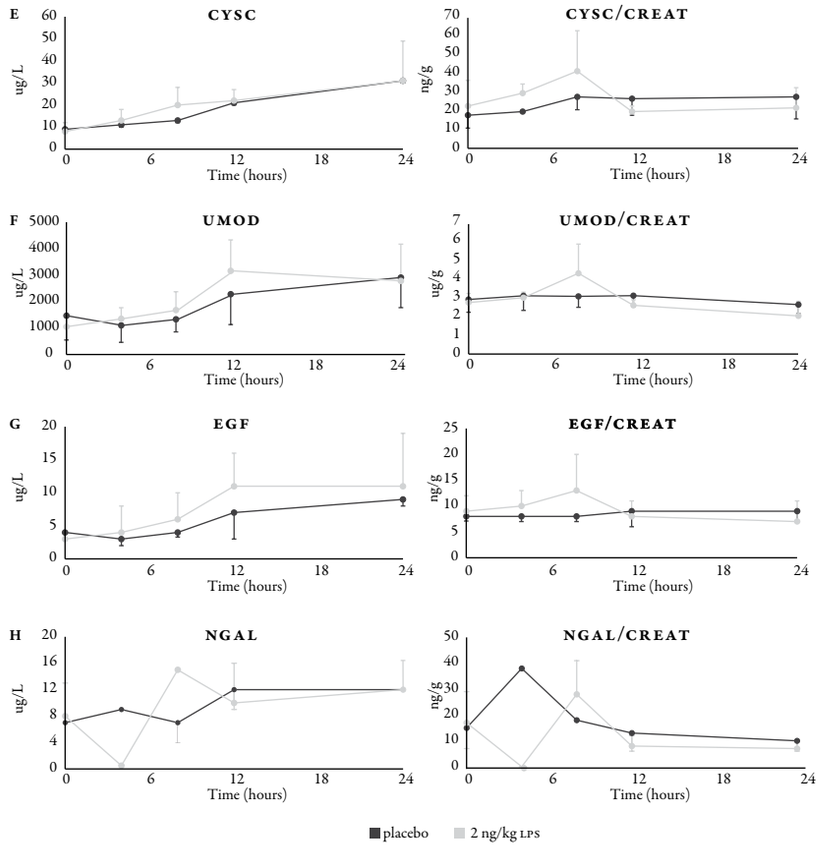


Figure 3. Urinary concentration of renal injury markers B2MG (A), KIM1 (B), OPN (C), NAG (D), CysC (E), UMOD (F), EGF (G) and NGAL (H), in absolute values (left panels), and corrected for urinary creatinine concentration (right panels) ± SD (error bars).



LPS administration was at T=0. For readability, only the standard deviations of the two extremes are shown.

Fig. 3. (continued)



LPS administration was at T=0. For readability, only the standard deviations of the two extremes are shown.

REFERENCES

1 Dillingh MR, van Poelgeest EP, Malone KE, et al. Characterization of inflammation and immune cell modulation induced by low-dose LPS administration to healthy volunteers. Journal of Inflammation-London 2014;11.

2 Andreasen AS, Krabbe KS, Krogh-Madsen R, Taudorf S, Pedersen BK, Moller K. Human endotoxemia as a model of systemic inflammation. Curr Med Chem 2008;15:1697-705.

3 Kox M, de KS, Pompe JC, et al. Differential *ex vivo* and *in vivo* endotoxin tolerance kinetics following human endotoxemia. Crit Care Med 2011;39:1866-70.

4 van der Poll T, Coyle SM, Moldawer LL, Lowry SF. Changes in endotoxin-induced cytokine production by whole blood after *in vivo* exposure of normal humans to endotoxin. J Infect Dis 1996;174:1356-60.

5 Schouten M, Wiersinga WJ, Levi M, van der Poll T. Inflammation, endothelium, and coagulation in sepsis. J Leukoc Biol 2008;83:536-45.

6 Cohen J. The immunopathogenesis of sepsis. Nature 2002;420:885-91.

7 Jilma B, Blann A, Pernerstorfer T, et al. Regulation of adhesion molecules during human endotoxemia. No acute effects of aspirin. Am J Respir Crit Care Med 1999;159:857-63.

8 Graninger M, Marsik C, Dukic T, Wagner OF, Blann AD, Jilma B. Enalapril does not alter adhesion molecule levels in human endotoxemia. Shock 2003;19:448-51.

9 Szmitko PE, Wang CH, Weisel RD, Jeffries GA, Anderson TJ, Verma S. Biomarkers of vascular disease linking inflammation to endothelial activation - Part II. circulation 2003;108:2041-8.

10 Decaterina R, Libby P, Peng HB, et al. Nitric-Oxide Decreases Cytokine-Induced Endothelial Activation - Nitric-Oxide Selectively Reduces Endothelial Expression of Adhesion Molecules and Proinflammatory Cytokines. Journal of Clinical Investigation 1995;96:60-8.

11 Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction - A marker of atherosclerotic risk. Arteriosclerosis Thrombosis and Vascular Biology 2003;23:168-75.

12 rajendran P, Rengarajan T, Thangavel J, et al. The Vascular Endothelium and Human Diseases. International Journal of Biological Sciences 2013;9:1057-69.

13 Segal MS, Baylis C, Johnson RJ. Endothelial health and diversity in the kidney. Journal of the American Society of Nephrology 2006;17:323-4.

14 Koyner JL, Vaidya VS, Bennett MR, et al. Urinary biomarkers in the clinical prognosis and early detection of acute kidney injury. Clin J Am Soc Nephrol 2010;5:2154-65.

15 Nguyen MT, Devarajan P. Biomarkers for the early detection of acute kidney injury. Pediatr Nephrol 2008;23:2151-7.

16 Vaidya VS, Waikar SS, Ferguson MA, et al. Urinary biomarkers for sensitive and specific detection of acute kidney injury in humans. Clin Transl Sci 2008;1:200-8.

17 Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. Ann Intern Med 1999;130:461-70.

18 Levey AS, Stevens LA, Schmid CH, et al. A new equation to estimate glomerular filtration rate. Ann Intern Med 2009;150:604-12.

19 Brott DA, Adler SH, Arani R, Lovick SC, Pinches M, Furlong ST. Characterization of renal biomarkers for use in clinical trials: biomarker evaluation in healthy volunteers. Drug Des Devel Ther 2014;8:227-37.

20 Myers CL, Wertheimer SJ, Schembri-King J, Parks T, Wallace RW. Induction of ICAM-1 by TNF-alpha, IL1 beta, and LPS in human endothelial cells after downregulation of PKC. Am J Physiol 1992;263:C767-C72.

21 Kansas GS. Selectins and their ligands: current concepts and controversies. Blood 1996;88:3259-87.

22 de Fougerolles AR, Springer TA. Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. J Exp Med 1992;175:185-90.

23 Patey N, Vazeux R, Canioni D, Potter T, Gallatin WM, Brousse N. Intercellular adhesion molecule-3 on endothelial cells. Expression in tumors but not in inflammatory responses. Am J Pathol 1996;148:465-72.

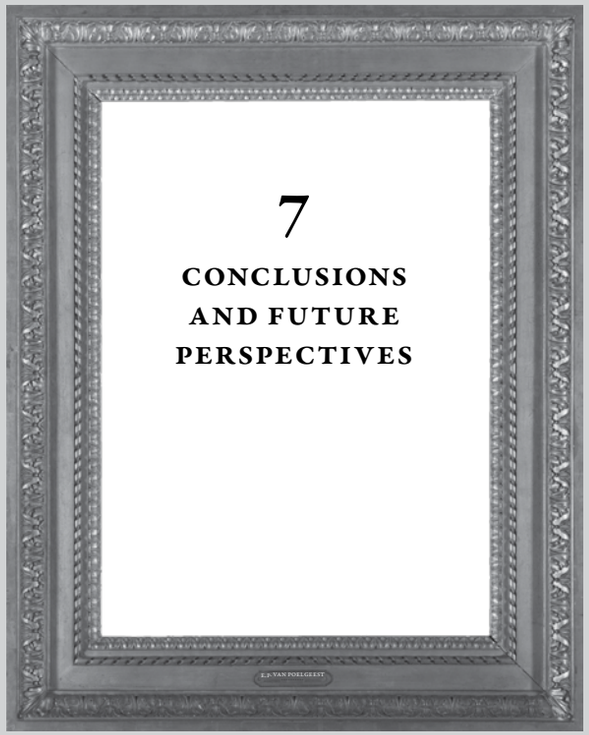
24 Collins RG, Velji R, Guevara NV, Hicks MJ, Chan L, Beaudet AL. P-Selectin or intercellular adhesion molecule (ICAM)-1 deficiency substantially protects against atherosclerosis in apolipoprotein E-deficient mice. J Exp Med 2000;191:189-94.

25 Cybulsky MI, Iiyama K, Li H, et al. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. J Clin Invest 2001;107:1255-62.

26 Chen FT, Liu YC, Yang CM, Yang CH. Anti-inflammatory effect of the proteasome inhibitor bortezomib on endotoxin-induced uveitis in rats. Invest Ophthalmol Vis Sci 2012;53:3682-94.

27 Zhu S, Xu X, Liu K, Gu Q, Yang X. PAPep, a small peptide derived from human pancreatitis-associated

protein, attenuates corneal inflammation *in vivo* and *in vitro* through the IKK alpha/beta/IkappaBalpha/NF-kappaB signaling pathway. Pharmacol Res 2015;102:113-22.
28 Yoshida T, Yamashita M, Iwai M, Hayashi M. Endothelial Kruppel-Like Factor 4 Mediates the Protective Effect of Statins against Ischemic aki. J Am Soc Nephrol 2016;27:1379-88.
29 Fijen JW, Tulleken JE, Kobold AC, et al. Inhibition of p38 mitogen-activated protein kinase: dose-dependent suppression of leukocyte and endothelial response after endotoxin challenge in humans. Crit Care Med 2002;30:841-5.
30 Guerrot D, Bellien J. Editorial: renal endothelial dysfunction: evolving concepts and perspectives. Cardiovasc Hematol Disord Drug Targets 2014;14:1-2.
31 van Eijk LT, Pickkers P, Smits P, Bouw MP, van der Hoeven JG. Severe vagal response after endotoxin administration in humans. Intensive Care Med 2004;30:2279-81.
32 Heemskerk S, Pickkers P, Bouw MP, et al. Upregulation of renal inducible nitric oxide synthase during human endotoxemia and sepsis is associated with proximal tubule injury. Clin J Am Soc Nephrol 2006;1:853-62.
33 Zijlstra JG, Tulleken JE, Ligtenberg JJ, de BP, van der Werf TS. p38-MAPK inhibition and endotoxin induced tubular dysfunction in men. J Endotoxin Res 2004;10:402-5.
34 van ML, Moerland M, Cohen AF, Burggraaf J. Urinary kidney biomarkers for early detection of nephrotoxicity in clinical drug development. Br J Clin Pharmacol 2014;77:947-57.
35 Hoffmann D, Fuchs TC, Henzler T, et al. Evaluation of a urinary kidney biomarker panel in rat models of acute and subchronic nephrotoxicity. Toxicology 2010;277:49-58.
36 Amin RP, Vickers AE, Sistare F, et al. Identification of putative gene based markers of renal toxicity. Environ Health Perspect 2004;112:465-79.
37 O'Regan A, Berman JS. Osteopontin: a key cytokine in cell-mediated and granulomatous inflammation. Int J Exp Pathol 2000;81:373-90.
38 Madsen KM, Zhang L, Abu Shamat AR, Siefgried S, Cha JH. Ultrastructural localization of osteopontin in the kidney: induction by lipopolysaccharide. J Am Soc Nephrol 1997;8:1043-53.
39 Ouchi M, Suzuki T, Hashimoto M, et al. Urinary N-acetyl-beta-D-glucosaminidase levels are positively correlated with 2-hr plasma glucose levels during oral glucose tolerance testing in prediabetes. J Clin Lab Anal 2012;26:473-80.
40 Bagshaw SM, Langenberg C, Haase M, Wan L, May CN, Bellomo R. Urinary biomarkers in septic acute kidney injury. Intensive Care Med 2007;33:1285-96.
41 Wood SM, Shah SS, Steenhoff AP, Meyers KE, Kaplan BS, Rutstein RM. Tenofovir-associated nephrotoxicity in two HIV-infected adolescent males. AIDS Patient Care STDS 2009;23:1-4.
42 Han WK, Waikar SS, Johnson A, et al. Urinary biomarkers in the early diagnosis of acute kidney injury. Kidney Int 2008;73:863-9.
43 Shinke H, Masuda S, Togashi Y, et al. Urinary kidney injury molecule-1 and monocyte chemotactic protein-1 are noninvasive biomarkers of cisplatin-induced nephrotoxicity in lung cancer patients. Cancer Chemother Pharmacol 2015;76:989-96.
44 Vaidya VS, Waikar SS, Ferguson MA, et al. Urinary biomarkers for sensitive and specific detection of acute kidney injury in humans. Clin Transl Sci 2008;1:200-8.
45 Bruning T, Sundberg AG, Birner G, et al. Glutathione transferase alpha as a marker for tubular damage after trichloroethylene exposure. Arch Toxicol 1999;73:246-54.
46 Sundberg A, Appelkvist EL, Dallner G, Nilsson R. Glutathione transferases in the urine: sensitive methods for detection of kidney damage induced by nephrotoxic agents in humans. Environ Health Perspect 1994;102 Suppl 3:293-6.
47 Patel PN, Shah RY, Ferguson JF, Reilly MP. Human experimental endotoxemia in modeling the pathophysiology, genomics, and therapeutics of innate immunity in complex cardiometabolic diseases. Arterioscler Thromb Vasc Biol 2015;35:525-34.



7

CONCLUSIONS AND FUTURE PERSPECTIVES

Although statins have greatly improved cardiovascular morbidity and mortality over the last decades [1], over 2/3 of patients at (very) high risk for cardiovascular disease suffer from life-threatening events despite being on statin therapy [2,3]. Lowering this risk with novel drugs would therefore have great impact on contemporary atherosclerotic cardiovascular disease management. Thematically organized, these drugs reduce cardiovascular risk by decreasing (1) increased LDL-C levels, (2) inflammation, and/or (3) dysfunctional endothelial barrier function resulting in subendothelial cholesterol accumulation and subsequent atheroma formation. In this thesis, we describe the first clinical studies with novel compounds based on themes 1 and 2 (Chapters 2 and 5), including the methodology required for drug evaluation (Chapter 4), and present the methodology that may be useful for the clinical development future compounds targeting the endothelium (theme 3, Chapter 6).

TARGETING RESIDUAL LIPID RISK, PCSK9 INHIBITION

There is a clear unmet need for novel potent lipid-lowering drugs [4]. PCSK9 is a protein that is responsible for LDL-R degradation within hepatocytes [5,6]. Inhibition of this enzyme increases recycling of LDL-Rs to the hepatocyte cell surface, and thereby increases the clearance of LDL-C from the circulation. Pharmacological PCSK9 inhibition holds promise as a long-awaited breakthrough in the treatment of hypercholesterolemia. Santaris Pharma A/S developed SPC5001, an LNA-based PCSK9-targeted antisense oligonucleotide, to treat hypercholesterolemia. In a first-in-human double-blind, placebo-controlled, single dose-escalation study, described in **CHAPTER 2**, we explored the tolerability, pharmacokinetics and pharmacodynamics of three weekly subcutaneous injections SPC5001 in healthy volunteers with moderately elevated LDL-C concentrations. Proof-of-pharmacology was achieved: even at low doses (up to 5 mg/kg), SPC5001 reduced plasma PCSK9 and LDL-C, with a maximal decrease in PCSK9 concentration of approximately 50% and a reduction in LDL-C of maximally 25% compared to baseline. Due to safety reasons, evaluation of the pharmacodynamic effect of SPC5001 was only limited: SPC5001 dose-dependently increased serum creatinine levels peaking 1.5 week after the final injection, and returning to baseline within 1 week. This increase was only moderate in most participants, amounting approximately 15% over baseline at the highest dose, but severe in one participant in the 5 mg/kg dose group. This subject developed acute kidney injury based on biopsy-proven acute tubular necrosis. In **CHAPTER 3**, a detailed case report of this volunteer is presented. The adverse effects on renal function prompted discontinuation of the clinical trial, and intensification of renal monitoring of the study participants. Besides serum creatinine and urea, considered the golden standard for diagnosing acute kidney injury, but known for their non-specificity and delayed capacity in detecting early subtle signs of kidney injury [7], a panel of promising novel urinary kidney injury biomarkers was measured to gain insight in their potential to detect subtle signs of kidney injury, and to pinpoint the precise site of injury within the nephron [8]. This is valuable information as the clinical applicability of these novel kidney injury biomarkers is only limited: most markers are not fully validated yet, likely due to the fact that the available literature is based on observational data with great heterogeneity in patient characteristics, and variability in study designs and data normalization approaches (e.g. concentration of the biomarker, or expressed as absolute excretion per hour, or corrected for urinary creatinine excretion) [9]. We demonstrated that a selected panel of exploratory novel urinary kidney injury markers allows diagnosing acute kidney injury several days earlier than the traditional kidney injury markers.

SPC5001 kidney toxicity was unexpected as antisense oligonucleotides are widely explored in clinical trials, and generally considered non-toxic for the kidney, even at high concentrations [10]. Extensive preclinical toxicology testing in nonhuman primates did not point toward SPC5001-induced adverse renal effects (data on file at Santaris A/S and [11]). Moreover, genetically low PCSK9 levels [12] and PCSK9 inhibition per se [13,14] are devoid of adverse renal effects. Although our data do not explain the exact cause of SPC5001-related renal toxicity, and are only based on one particular antisense oligonucleotide compound, our findings do increase the awareness for the nephrotoxic effects of ASOs and underline the need for more thorough monitoring of kidney function in the clinical development and utilization of ASOs. Indeed, publication on the observed renal toxicity of SPC5001 (**CHAPTERS 2** and **3**) has already led to an increase in stringency of renal safety screening of LNA oligonucleotides as determined during the 2013 edition of the DIA/FDA Oligonucleotide-Based Therapeutic Conference, a platform for pharmaceutical companies and the regulatory authority. Further mechanistic studies are in progress to gain detailed insight in factors causing SPC5001-related toxicity to the kidney [15].

Besides the adverse effects on the kidney, SPC5001 caused inflammatory injection site reactions. In 44% of the SPC5001 treated subjects, ISRs were of mild to moderate severity and caused significant discomfort to a subset of the volunteers. In one female, a generalized maculopapular rash developed 1 week after the final dose. In general, the ISRs persisted for several days to weeks and then diminished in intensity. However, in one female, subcutaneous skin atrophy developed,

which was present at the final visit 2.5 months after dosing. Also, in six out of eight SPC5001-treated subjects skin hyperpigmentation was present at the last follow-up visit at 2.5 months. The observation of SPC5001-induced ISRs is in line with previous reports in literature that indicate that oligonucleotides have the potential to induce ISRs [16]. However, SPC5001 is a new-generation oligonucleotide, engineered with increased potency and duration of action compared to earlier generation ASOs, and therefore holding promise for reduced oligonucleotide-induced ISR incidence and severity [17-19]. Preclinical results with were SPC5001 promising: subcutaneous administration resulted in only minimal injection site effects in a small subset of nonhuman primates (data on file at Santaris A/S). Additional studies into the pathophysiology of SPC5001-induced inflammatory skin reactions are desired. An important challenge to overcome is that these skin effects apparently cannot be studied reliably in commonly used animal models [20]. It would be interesting to explore whether this issue can be overcome by application of a humanized mouse model of human immunity [21] combined with human skin transplants. If the ongoing attempts in engineering oligonucleotides for oral administration [22] prove success, this delivery approach is obviously preferred.

The unfavourable safety profile of SPC5001, prohibiting its further clinical development, was disappointing as it seems that the emerging class of PCSK9 inhibitors will bring the long-awaited breakthrough in pharmacological lipid lowering. This offers opportunities for high-risk patients for whom effective treatment options have been lacking for decades. Two fully humanised monoclonal antibodies directed at PCSK9 (Evolocumab and Alirocumab) have recently been implemented in the treatment of patients at high cardiovascular risk, intolerant to statins or unable to reach their LDL-C target with aggressive statin treatment [23]. These drugs exhibit a favourable safety and tolerability profile, while lowering LDL-C with potency well beyond statins (~50-70% and ~30% LDL-C reduction in individuals without and with familial hypercholesterolemia, respectively) [24]. In a recent landmark trial [25] with one of these monoclonal antibodies, Evolocumab, evidence was provided that PCSK9 inhibition combined with aggressive statin treatment confers additional cardiovascular risk reduction. Besides monoclonal antibodies inhibiting PCSK9, other evolving PCSK9-inhibiting approaches include mimetic peptides, small molecule inhibitors and gene silencing drugs. Of these, Inclisiran (ALN-PCS) showed promising results in recent trials [26,27]: a single 300 mg injection significantly reduced PCSK9 by 75% and LDL-C by 51%, an effect that lasted at least 6 months without safety concerns. Whereas both Inclisiran and SPC5001 are gene silencing drugs, Inclisiran has been engineered to specifically target hepatocytes [28], the major source of PCSK9 [29], without affecting extrahepatic PCSK9 function. Theoretically, this offers an advantage compared to monoclonal antibodies blocking circulating PCSK9, as PCSK9 physiology in tissues other than the liver is incompletely understood [30]. Nevertheless, all data collected so far with PCSK9 inhibitors have demonstrated that PCSK9 inhibition is probably safe [31]. Given these promising data, it is likely that in the near future PCSK9 inhibiting drugs will play an increasingly important role in lowering residual cardiovascular risk.

A METHODOLOGICAL TOOL IN ANTI ATHEROSCLEROTIC DRUG DEVELOPMENT

Besides lowering residual lipid risk, strategies to reduce inflammatory burden hold promise to lower cardiovascular morbidity and mortality: half of the patients that develop cardiovascular events do so with low cholesterol levels [32], but an elevated inflammatory burden [33]. Indeed, it is well-established that inflammation is the driving force behind all pathophysiological phases of atherosclerotic disease [34]. Therefore, anti-inflammatory drugs may have important consequences for contemporary atherosclerosis treatment. Whereas evaluation of the intended pharmacology of lipid-lowering drugs in healthy volunteers (**CHAPTER 2**) is uncomplicated due to the fact that elevated LDL-C levels are present in otherwise healthy individuals, proper evaluation of a drug's anti-inflammatory effects in healthy volunteers requires an inflammatory disease model. The human endotoxemia model is a well-established experimental model for studying inflammation in healthy volunteers [35,36]. In this model, exogenous administration of standardized preparations of lipopolysaccharide (LPS) activates innate immunity through TLR4 signaling. A relatively high dose of LPS (exceeding 2 ng/kg) is generally applied in experimental endotoxemia. Using such high LPS doses, however, is unnecessarily burdensome for volunteers and induces a supraphysiologic, sepsis-like immune response, accompanied by counter-regulatory (endocrine) responses, potentially masking beneficial immune-modulating drug effects in low-grade systemic inflammatory conditions. Thorough characterization of inflammatory effects of experimental low-dose endotoxemia was lacking. We demonstrated that administration of a single low dose of LPS (0.5, 1 and 2 ng/kg body weight) to healthy volunteers robustly increased acute phase reactants and pro-inflammatory cytokines (TNFα, IL6 and IL8), as described in **CHAPTER 4**. Besides the effects of low-dose endotoxemia on the acute phase inflammatory response, we aimed to characterize its effects on endothelial function, since LPS doses exceeding 2 ng/kg

had been demonstrated to activate the endothelium, leading to endothelial dysfunction [37,38]. Systematically collected and quantitative data on endotoxin-induced activation of the human endothelium, however, was not available. Such data would be valuable because a human *in vivo* model with controlled and transient activation of the endothelium could qualify the human endotoxin challenge as a pharmacodynamic model for the early clinical development of new drugs (and non-pharmacological interventions), developed to improve or maintain integrity of the endothelium. We showed that low doses of LPS dose-dependently increased the circulating levels of the endothelial activation markers P- and E-Selectin, VCAM1, ICAM1, and TM, as presented in **CHAPTER 6**.

Since the LPS challenge can be an important methodological tool in early phase clinical drug development, we investigated the effects of LPS administration on renal safety. This is especially relevant since observational data suggested a possible association between administration of LPS doses ≥2 ng/kg and acute kidney injury [39,40]. Detailed insight in the LPS concentration-effect relationship, in terms of the magnitude, variability and timing of the renal response, however, was lacking. In **CHAPTER 6,** we describe the effects of low-dose endotoxemia, not only on traditional kidney injury biomarkers, but also on an extensive panel of exploratory and highly sensitive novel kidney injury markers. We demonstrated that low-dose LPS administration did not cause adverse renal effects. In the highest LPS dose (2 ng/kg) group, however, significant increases in urinary concentrations of B2MG, KIM1 and OPN were observed in the time interval 8-24hrs after LPS administration. Although this may be indicative of subtle tubular damage, kidney function was unaffected and correction for urinary volume blunted these effects that were only limited compared to AKI of various other etiologies reported in the literature [41,42]. Therefore, it was concluded that the renal effects of low-dose LPS administration (if any) were of borderline relevance, underlining the renal safety when considering the LPS challenge as a methodological tool for future pharmacology studies.

When considering the low-dose endotoxemia model as a methodological tool in clinical drug testing, another issue to address is LPS hyporesponsiveness, the phenomenon that the inflammatory effects upon prior *in vivo* LPS challenging are temporarily attenuated within individuals after subsequent *ex vivo* LPS challenge testing [43]. The exact time course of this feature and relation to LPS dose level, however, was unknown. We expanded the knowledge on this topic by demonstrating that *in vivo* LPS administration dose-dependently induced a period of hyporesponsiveness in the *ex vivo* LPS-induced cytokine release, with maximal hyporesponsiveness observed at 6 hours, but lasting no longer than 12 hours. Based on these findings, described in **CHAPTER 4**, we concluded that a combination of a low-dose *in vivo* LPS challenge combined with (repeated) *ex vivo* LPS challenges performed from 12 hours after *in vivo* LPS challenging is a feasible approach for future clinical studies exploring immune-modulating drug effects. Moreover, we demonstrated the potential use of the model for controlled modulation of the human vasculature, which is valuable information, as a human model of atherosclerotic disease is currently not available. Interestingly in this context, preliminary data indicate that low-dose endotoxemia may be informative for studying atherosclerosis [44] since it acutely evokes a systemic pro-inflammatory and metabolic condition in close resemblance to that observed chronically in individuals with atherosclerosis. Indeed, the similarities between the effects of endotoxemia and the observations in animal models and individuals with atherosclerotic disease are striking. First of all, there is a wealth of evidence indicating that there is a link between the pro-inflammatory cytokines and chemokines, and endothelial activation/dysfunction markers we have demonstrated to be induced by low-dose endotoxemia (**CHAPTERS 4-6**) and in atherosclerosis. For instance, in patients with coronary artery disease, circulating levels of adhesion molecules and selectins are elevated [45]. Increased P-Selectin plasma levels are present in patients with unstable angina compared to patients with stable coronary artery disease [46-48]. In apolipoprotein E-deficient mice, deficiency of P-Selectin or ICAM1 was protective against atherosclerosis [49], and blocking P-Selectin in various animal models of atherosclerosis reduced post myocardial infarct size and ischemia-reperfusion injury [50]. In a large study (N=544) involving patients with non-ST-segment elevation myocardial infarction, a selective P-selectin inhibitor reduced myocardial damage during a percutaneous coronary intervention (-23.8% reduction in peak troponin I compared to placebo, p=0.05) [51]. In healthy volunteers, a synthetic p38 mitogen-activated protein kinase inhibitor prevented endothelial activation as measured by E-selectin and ICAM1 release after infusion of high-dose (4 ng/kg) LPS [52]. Also, in endothelial regions covering fatty streaks and (human) atheroma, P-selectin [46-48] and CXCL10 is highly expressed [53]. TNFα is expressed in the majority of atherosclerotic specimens, whereas it is absent in non-atherosclerotic sections [54]. In animals, CXCL10 has been shown to play an important role in atherogenesis, and blocking it reduces atherosclerosis [55].

Another feature that endotoxemia and atherosclerosis have in common is TLR4 signaling. TLR4 not only recognizes LPS (its typical exogenous ligand), but also various endogenous ligands that are upregulated in atherosclerosis, including modified LDL [56,57]. This receptor appears important in the differentiation from macrophages to foam cells [57], a cardinal step in atherogenesis. TLR4 expression

is highly upregulated, both *in vitro* in endothelial cells upon stimulation by pro-inflammatory cytokines [58], and *in vivo* in atherosclerotic vessels compared to non-atherosclerotic vessels [59]. In patients with unstable angina and acute myocardial infarction, an increase in levels of circulating TLR4 positive monocytes has been observed [54]. Pharmacological inhibition of TLR4 signaling may be an effective approach in atherosclerotic cardiovascular disease as animal studies show that TLR4 knock-out mice crossed with atheroprone apolipoprotein E deficient mice have significantly reduced aortic atherosclerosis, circulating levels of pro-inflammatory cytokines and plaque lipid content compared to control mice with comparable serum cholesterol levels [60,61].

TARGETING RESIDUAL INFLAMMATION RISK, TLR4 SIGNALING BLOCKADE

The low-dose endotoxemia model was applied as a methodological tool for demonstrating proof-of-pharmacology of NI-0101, a monoclonal antibody blocking TLR4 signalling (**CHAPTER 5**). This was a 2-part Phase 1 PK/PD guided study. In Part 1, single ascending doses of NI-0101 up to 15 mg/kg were well tolerated without safety concerns. A wide range of plasma concentrations was covered, up to those theoretically reflecting a potential therapeutic effect on inflamed tissues, as predicted by PK/PD modeling. Importantly, there were no indications that NI-0101 administration increased the risk for (Gram negative) infections. This is consistent with the safety reports from clinical trials performed with other molecules targeting TLR4 [62,63]. This is also in line with reports on primary immunodeficiency associated with impaired TLR4 signalling resulting from mutations in MYD88- or TRIF-related genes [64,65], indicating that blocking TLR signaling is not related to an increased susceptibility to infections. Probably, this can be explained by redundancy of the immune system, which is supported by the observations of TLR4-independent inflammatory responses to LPS [66,67].

Besides its good safety and tolerability, NI-0101 demonstrated a favourable pharmacokinetic and pharmacodynamic profile, characterized by a long half-life of approximately 10 days, which supports infrequent dosing of the drug. NI-0101 doses exceeding 1 mg/kg near-completely (≥98%) blocked the cytokine response in blood following an *in vivo* LPS challenge. The duration of complete inhibition was dose-dependent and lasted at least 10 weeks at the highest dose tested (15 mg/kg). As expected by the PK/PD simulations and IC_{50} data reported from *in vitro* experiments (Novimmune internal reports), the duration of cytokine inhibition was dependent on the FcγRIIa genotype of the study participants. Whether this translates to differences on clinical response will be addressed in future clinical trials to be performed with NI-0101.

The main objective of Part 2 of the trial was to compare the concentration-effect relationship for NI-0101 *ex vivo* and *in vivo* (by application of the human endotoxemia model). The NI-0101 starting dose of 0.01 mg/kg was selected based on *ex vivo* cytokine release inhibition observed in part 1, with an anticipated inhibition of *in vivo* LPS-induced cytokine production of at least 75% for a duration of 3 days. The second NI-0101 dose of 0.25 mg/kg was intended to induce prolonged (i.e., up to 28 days) and marked (≥90%) inhibition of *in vivo* LPS-induced cytokine production. Volunteers received iv LPS either immediately at the end of NI-0101/placebo (expected T_{max} of NI-0101), or at a delayed point in time (22 or 40 days later). Even at low doses (0.01 or 0.25 mg/kg), NI-0101 completely abrogated the typical clinical LPS-induced clinical signs and symptoms (influenza-like illness, increase in body temperature and heart rate), not only when administered shortly preceding the LPS challenge, but also when the LPS challenge was conducted three weeks after drug administration. Comparable results have been shown for Eritoran [68] and IC-14 [69]. Although marked differences in cytokine response exist between *in vivo* and *ex vivo* LPS challenges [70], NI-0101 effect size and duration as estimated by an *in vivo* and an *ex vivo* LPS challenge appeared well comparable. Therefore, it was concluded that the *ex vivo* LPS challenge may be a suitable and minimally invasive surrogate for the *in vivo* LPS challenge. This is obviously important for future drug studies as repeated *ex vivo* testing over time is only minimally invasive for the study participants and convenient for the investigator. This trial demonstrated that basic cellular/molecular knowledge can be translated into a rational clinical trial design. Moreover, based on the generated data, a PK/PD model was built to guide future clinical development of NI-0101, providing a tool to rationally select the dose level and regimen for phase 2 studies.

Currently, NI-0101 is being evaluated in patients with rheumatoid arthritis. TLR4 has been proposed to play a role in RA pathogenesis [71,72]. Besides important in RA pathophysiology, TLR4 ligand presence and/or excessive and/or inappropriate TLR4 signalling contributes to the pathophysiology of relatively common conditions (e.g. acute kidney injury [73,74] and diabetic nephropathy [75]), and rare auto-inflammatory syndromes [76], all in need of novel effective therapeutic options. Therefore, TLR4 inhibition may be implemented in clinical practice well beyond the treatment of rheumatoid arthritis. Given the wealth of data assuming a pathophysiological role of TLR4 signalling in atherosclerosis as discussed earlier, this may also be a promising target condition for NI-0101. Indeed, a recent meta-analysis showed that inhibition of one of the main inflammatory

cytokines downstream of the TLR4, TNFα, is associated with a marked reduction in the risk of developing coronary artery disease in RA patients [77]. Therefore, it would be interesting to prospectively evaluate atherosclerotic plaque formation in RA patients on NI-0101 treatment in the future. Whether broad systemic anti-inflammatory drug interventions will acquire an important role in atherosclerosis treatment in patients without chronic inflammatory disease in the long-term, however, is questionable: after 4 decades of the inflammation theory in atherosclerosis, strong evidence for the hypothesis that targeting inflammation systemically lowers cardiovascular events in high-risk patients is still lacking. Two large ongoing trials with canacinumab [78], an anti IL1β monoclonal antibody, and methotrexate [79], an anti-inflammatory drug that reduces TNFα, IL6, and CRP levels, respectively, address this important issue. The results of these trials are expected to be published within the next year. Theoretically, a more specific treatment is preferred over broad systemic anti-inflammatory interventions as atheroma formation is not randomly localized within the arterial vascular tree, but is situated preferentially in distinct atheroprone areas. Depending on disturbed blood flow and magnitude of endothelial shear stress, considerable site-specific differences in endothelial cell phenotype are observed that in turn either promote or suppress atheroma formation [32,80,81]. In this context, basic molecular insights have led to the development of various drugs currently under active development, specifically targeting atheroma-specific areas within the vascular tree with sophisticated drug delivery techniques [82,83].

SUMMARY AND FUTURE PERSPECTIVES

In this thesis, two first-in-human clinical studies with novel drugs were presented (**CHAPTERS 2**, **3** and **5**). In healthy volunteers with elevated LDL-C, we demonstrated proof-of-pharmacology of PCSK9 inhibitor SPC5001 in lowering LDL-C levels, but toxicity of this compound prevented its further clinical development (**CHAPTERS 2** and 3). Other PCSK9 inhibitors seem safe and well-tolerated, while lowering LDL-C levels with unprecedented robustness. Therefore, PCSK9 inhibitors are expected to cause a long-awaited breakthrough in the treatment of hypercholesterolemia.

In order to demonstrate proof-of-pharmacology in healthy volunteers of the second drug in this thesis (**CHAPTER 5**), anti-inflammatory drug NI-0101, a TLR4 inhibitor, we demonstrated that the low-dose experimental endotoxemia model results in the controlled induction of inflammation (**CHAPTER 4**) and triggering of the vasculature (**CHAPTER 6**) in otherwise healthy individuals, with close resemblance to the situation present chronically in atherosclerotic patients. As such, we argue that this model may be suitable for evaluation of future potentially atheroprotective drugs. Of course, one should be aware that the immune response in atherosclerosis comprises a combination of innate, adaptive and humoral immune responses in which also TLRs other than TLR4 play important roles [84]. On the other hand, low-dose endotoxemia induces transient inflammatory and metabolic changes that resemble remarkably well the changes observed chronically in inflammatory cardiovascular disease risk states such as atherosclerosis [44]. Importantly, the low-dose endotoxemia model involves TLR4-, MYD88-, and TRIF-driven inflammatory pathways, and as such is a relevant model for all future drugs designed to modulate a target driven by these inflammatory cascades.

We face an exciting era in targeting residual risk reduction in atherosclerosis treatment in which our contemporary armamentarium will be augmented by highly potent LDL-C lowering drugs and possibly also anti-inflammatory drugs. Early phase drug development studies targeting inflammation could benefit from the low-dose endotoxemia model as a methodological tool.

REFERENCES

1 Law MR, Wald NJ, Rudnicka AR. Quantifying effect of statins on low density lipoprotein cholesterol, ischAEmic heart disease, and stroke: systematic review and meta-analysis. BMJ 2003;326:1423.
2 Ahn CH, Choi SH. New drugs for treating dyslipidemia: beyond statins. Diabetes Metab J 2015;39:87-94.
3 Klingenberg R, Hansson GK. Treating inflammation in atherosclerotic cardiovascular disease: emerging therapies. eur Heart J 2009;30:2838-44.
4 George M, Selvarajan S, Muthukumar R, Elangovan S. Looking into the crystal ball-upcoming drugs for dyslipidemia. J Cardiovasc Pharmacol Ther 2015;20:11-20.
5 Wang Y, Huang Y, Hobbs HH, Cohen JC. Molecular characterization of proprotein convertase subtilisin/kexin type 9-mediated degradation of the LDL-R. J Lipid Res 2012;53:1932-43.
6 Horton JD, Cohen JC, Hobbs HH. PCSK9: a convertase that coordinates ldl catabolism. J Lipid Res 2009;50 Suppl:S172-7.
7 van Meer L, Moerland M, Cohen AF, Burggraaf J. Urinary kidney biomarkers for early detection of nephrotoxicity in clinical drug development. Br J Clin Pharmacol 2014;77:947-57.
8 Vaidya VS, Ferguson MA, Bonventre JV. Biomarkers of acute kidney injury. Annu Rev Pharmacol Toxicol 2008;48:463-93.
9 Coca SG, Yalavarthy R, Concato J, Parikh CR. Biomarkers for the diagnosis and risk stratification of acute kidney injury: a systematic review. Kidney Int 2008;73:1008-16.
10 Crooke ST. Antisense drug technology, principles, strategies and applications: CRC Press; 2007.
11 Lindholm MW, Elmen J, Fisker N, et al. PCSK9 LNA antisense oligonucleotides induce sustained reduction of ldl cholesterol in nonhuman primates. Mol Ther 2012;20:376-81.
12 Zhao Z, Tuakli-Wosornu Y, Lagace TA, et al. Molecular characterization of loss-of-function mutations in PCSK9 and identification of a compound heterozygote. Am J Hum Genet 2006;79:514-23.
13 Dias CS, Shaywitz AJ, Wasserman SM, et al. Effects of AMG 145 on low-density lipoprotein cholesterol levels: results from 2 randomized, double-blind, placebo-controlled, ascending-dose phase 1 studies in healthy volunteers and hypercholesterolemic subjects on statins. J Am Coll Cardiol 2012;60:1888-98.
14 Stein EA, Mellis S, Yancopoulos GD, et al. Effect of a monoclonal antibody to PCSK9 on ldl cholesterol. N Engl J Med 2012;366:1108-18.
15 Lindholm M. LNA oligonucleotide in vivo renal screening v2. 0 (Poster). DIA/FDA meeting on oligonucleotide therapeutics, Washington, September; 2013.
16 Hair P, Cameron F, McKeage K. Mipomersen sodium: first global approval. Drugs 2013;73:487-93.
17 Frazier KS. Antisense oligonucleotide therapies: the promise and the challenges from a toxicologic pathologist's perspective. Toxicol Pathol 2015;43:78-89.
18 Nair JK, Willoughby JL, Chan A, et al. Multivalent N-acetylgalactosamine-conjugated siRNA localizes in hepatocytes and elicits robust RNAi-mediated gene silencing. J Am Chem Soc 2014;136:16958-61.
19 Prakash TP, Graham MJ, Yu J, et al. Targeted delivery of antisense oligonucleotides to hepatocytes using triantennary N-acetyl galactosamine improves potency 10-fold in mice. Nucleic Acids Res 2014;42:8796-807.
20 Engelhardt JA. Predictivity of animal studies for human injection site reactions with parenteral drug products. Exp Toxicol Pathol 2008;60:323-7.
21 Shultz LD, Brehm MA, Garcia-Martinez JV, Greiner DL. Humanized mice for immune system investigation: progress, promise and challenges. Nat Rev Immunol 2012;12:786-98.
22 Tillman LG, Geary RS, Hardee GE. Oral delivery of antisense oligonucleotides in man. J Pharm Sci 2008;97:225-36.
23 Ito MK, Santos RD. PCSK9 Inhibition With Monoclonal Antibodies: Modern Management of Hypercholesterolemia. J Clin Pharmacol 2017;57:7-32.
24 Della Badia LA, Elshourbagy NA, Mousa SA. Targeting PCSK9 as a promising new mechanism for lowering low-density lipoprotein cholesterol. Pharmacol Ther 2016;164:183-94.
25 Sabatine MS, Giugliano RP, Keech AC, et al. Evolocumab and Clinical Outcomes in Patients with Cardiovascular Disease. N Engl J Med 2017.
26 Wang N, Tall AR. A New Approach to PCSK9 Therapeutics. circ Res 2017.
27 Ray KK, Landmesser U, Leiter LA, et al. Inclisiran in Patients at High Cardiovascular Risk with Elevated ldl Cholesterol. N Engl J Med 2017.
28 Fitzgerald K, White S, Borodovsky A, et al. A Highly Durable RNAi Therapeutic Inhibitor of PCSK9. N Engl J Med 2017;376:41-51.
29 Seidah NG, Mayer G, Zaid A, et al. The activation and physiological functions of the proprotein convertases. Int J Biochem Cell Biol 2008;40:1111-25.
30 Seidah NG, Awan Z, Chretien M, Mbikay M. PCSK9: a key modulator of cardiovascular health. circ Res 2014;114:1022-36.
31 Dadu RT, Ballantyne CM. Lipid lowering with PCSK9 inhibitors. Nat Rev Cardiol 2014;11:563-75.
32 Ross R. Atherosclerosis–an inflammatory disease. N Engl J Med 1999;340:115-26.
33 Ridker PM. Residual inflammatory risk: addressing the obverse side of the atherosclerosis prevention coin. eur Heart J 2016;37:1720-2.
34 Libby P. Inflammation in atherosclerosis. Nature 2002;420:868-74.
35 Andreasen AS, Krabbe KS, Krogh-Madsen R, Taudorf S, Pedersen BK, Moller K. Human endotoxemia as a model of systemic inflammation. Curr Med Chem 2008;15:1697-705.
36 Lowry SF. Human endotoxemia: a model for mechanistic insight and therapeutic targeting. Shock 2005;24 Suppl 1:94-100.
37 Jilma B, Blann A, Pernerstorfer T, et al. Regulation of adhesion molecules during human endotoxemia. No acute effects of aspirin. Am J Respir Crit Care Med 1999;159:857-63.
38 Graninger M, Marsik C, Dukic T, Wagner OF, Blann AD, Jilma B. Enalapril does not alter adhesion molecule levels in human endotoxemia. Shock 2003;19:448-51.
39 Zijlstra JG, Tulleken JE, Ligtenberg JJ, de BP, van der Werf TS. p38-MAPK inhibition and endotoxin induced tubular dysfunction in men. J Endotoxin Res 2004;10:402-5.
40 Heemskerk S, Pickkers P, Bouw MP, et al. Upregulation of renal inducible nitric oxide synthase during human endotoxemia and sepsis is associated with proximal tubule injury. Clin J Am Soc Nephrol 2006;1:853-62.
41 Bagshaw SM, Langenberg C, Haase M, Wan L, May CN, Bellomo R. Urinary biomarkers in septic acute kidney injury. Intensive Care Med 2007;33:1285-96.
42 Vaidya VS, Waikar SS, Ferguson MA, et al. Urinary biomarkers for sensitive and specific detection of acute kidney injury in humans. Clin Transl Sci 2008;1:200-8.
43 Kox M, de Kleijn S, Pompe JC, et al. Differential ex vivo and in vivo endotoxin tolerance kinetics following human endotoxemia. Crit Care Med 2011;39:1866-70.
44 Patel PN, Shah RY, Ferguson JF, Reilly MP. Human experimental endotoxemia in modeling the pathophysiology, genomics, and therapeutics of innate immunity in complex cardiometabolic diseases. Arterioscler Thromb Vasc Biol 2015;35:525-34.
45 Braunersreuther V, Mach F, Steffens S. The specific role of chemokines in atherosclerosis. Thromb HAEmost 2007;97:714-21.
46 Johnson-Tidey RR, McGregor JL, Taylor PR, Poston RN. Increase in the adhesion molecule P-selectin in endothelium overlying atherosclerotic plaques. Coexpression with intercellular adhesion molecule-1. Am J Pathol 1994;144:952-61.
47 Tenaglia AN, Buda AJ, Wilkins RG, et al. Levels of expression of P-selectin, E-selectin, and intercellular adhesion molecule-1 in coronary atherectomy specimens from patients with stable and unstable angina pectoris. Am J Cardiol 1997;79:742-7.
48 Jaumdally RJ, Varma C, Blann AD, MacFadyen RJ, Lip GY. Platelet activation in coronary artery disease: intracardiac vs peripheral venous levels and the effects of angioplasty. Chest 2007;132:1532-9.
49 Collins RG, Velji R, Guevara NV, Hicks MJ, Chan L, Beaudet AL. P-Selectin or intercellular adhesion molecule (AM)-1 deficiency substantially protects against atherosclerosis in apolipoprotein E-deficient mice. J Exp Med 2000;191:189-94.
50 Khan R, Spagnoli V, Tardif JC, L'Allier PL. Novel anti-inflammatory therapies for the treatment of atherosclerosis. Atherosclerosis 2015;240:497-509.
51 Tardif JC, Tanguay JF, Wright SR, et al. Effects of the P-selectin antagonist inclacumab on myocardial damage after percutaneous coronary intervention for non-ST-segment elevation myocardial infarction: results of the SELECT-ACS trial. J Am Coll Cardiol 2013;61:2048-55.
52 Fijen JW, Tulleken JE, Kobold AC, et al. Inhibition of p38 mitogen-activated protein kinase: dose-dependent suppression of leukocyte and endothelial response after endotoxin challenge in humans. Crit Care Med 2002;30:841-5.
53 Mach F, Sauty A, Iarossi AS, et al. Differential expression of three T lymphocyte-activating CXC chemokines by human atheroma-associated cells. J Clin Invest 1999;104:1041-50.
54 Frantz S, Ertl G, Bauersachs J. Mechanisms of disease: Toll-like receptors in cardiovascular disease. Nat Clin Pract Cardiovasc Med 2007;4:444-54.
55 Heller EA, Liu E, Tager AM, et al. Chemokine CXCL10 promotes atherogenesis by modulating the local balance of effector and regulatory T cells. circulation 2006;113:2301-12.
56 Miller YI, Viriyakosol S, Worrall DS, Boullier A, Butler S, Witztum JL. Toll-like receptor 4-dependent and -independent cytokine secretion induced by minimally oxidized low-density lipoprotein in macrophages. Arterioscler Thromb Vasc Biol 2005;25:1213-9.
57 Choi SH, Harkewicz R, Lee JH, et al. Lipoprotein accumulation in macrophages via toll-like receptor-4-dependent fluid phase uptake. circ Res 2009;104:1355-63.
58 Faure E, Thomas L, Xu H, Medvedev A, Equils O, Arditi M. Bacterial lipopolysaccharide and IFN-gamma induce Toll-like receptor 2 and Toll-like receptor 4 expression in

human endothelial cells: role of NF-kappa B activation. J Immunol 2001;166:2018-24.
59 Li H, Sun B. Toll-like receptor 4 in atherosclerosis. J Cell Mol Med 2007;11:88-95.
60 Bjorkbacka H, Kunjathoor VV, Moore KJ, et al. Reduced atherosclerosis in MyD88-null mice links elevated serum cholesterol levels to activation of innate immunity signaling pathways. Nat Med 2004;10:416-21.
61 Michelsen KS, Wong MH, Shah PK, et al. Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. Proc Natl Acad Sci U S A 2004;101:10679-84.
62 Rice TW, Wheeler AP, Bernard GR, et al. A randomized, double-blind, placebo-controlled trial of TAK-242 for the treatment of severe sepsis. Crit Care Med 2010;38:1685-94.
63 Barochia A, Solomon S, Cui X, Natanson C, Eichacker PQ. Eritoran tetrasodium (E5564) treatment for sepsis: review of preclinical and clinical studies. Expert Opin Drug Metab Toxicol 2011;7:479-94.
64 Picard C, Casanova JL, Puel A. Infectious diseases in patients with IraK-4, MyD88, NEMO, or IkappaBalpha deficiency. Clin Microbiol Rev 2011;24:490-7.
65 Brown KL, Falsafi R, Kum W, et al. Robust TLR4-induced gene expression patterns are not an accurate indicator of human immunity. J Transl Med 2010;8:6.
66 Hagar JA, Powell DA, Aachoui Y, Ernst RK, Miao EA. Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. Science 2013;341:1250-3.
67 Kayagaki N, Wong MT, Stowe IB, et al. Noncanonical inflammasome activation by intracellular LPS independent of TLR4. Science 2013;341:1246-9.
68 Lynn M, Rossignol DP, Wheeler JL, et al. Blocking of responses to endotoxin by E5564 in healthy volunteers with experimental endotoxemia. J Infect Dis 2003;187:631-9.
69 Verbon A, Dekkers PE, ten Hove T, et al. IC14, an anti-CD14 antibody, inhibits endotoxin-mediated symptoms and inflammatory responses in humans. J Immunol 2001;166:3599-605.
70 Dorresteijn MJ, Draisma A, van der Hoeven JG, Pickkers P. Lipopolysaccharide-stimulated whole blood cytokine production does not predict the inflammatory response in human endotoxemia. Innate Immun 2010;16:248-53.
71 Radstake TR, Roelofs MF, Jenniskens YM, et al. Expression of toll-like receptors 2 and 4 in rheumatoid synovial tissue and regulation by proinflammatory cytokines interleukin-12 and interleukin-18 via interferon-gamma. Arthritis Rheum 2004;50:3856-65.
72 Baillet A, Trocme C, Berthier S, et al. Synovial fluid proteomic fingerprint: S100A8, S100A9 and S100A12 proteins discriminate rheumatoid arthritis from other inflammatory joint diseases. Rheumatology (Oxford) 2010;49:671-82.
73 Zarbock A, Gomez H, Kellum JA. Sepsis-induced acute kidney injury revisited: pathophysiology, prevention and future therapies. Curr Opin Crit Care 2014;20:588-95.
74 Mudaliar H, Pollock C, Komala MG, Chadban S, Wu H, Panchapakesan U. The role of Toll-like receptor proteins (TLR) 2 and 4 in mediating inflammation in proximal tubules. Am J Physiol Renal Physiol 2013;305:F143-54.
75 Lin M, Yiu WH, Wu HJ, et al. Toll-like receptor 4 promotes tubular inflammation in diabetic nephropathy. J Am Soc Nephrol 2012;23:86-102.
76 Caso F, Rigante D, Vitale A, et al. Monogenic autoinflammatory syndromes: state of the art on genetic, clinical, and therapeutic issues. Int J Rheumatol 2013;2013:513782.
77 Barnabe C, Martin BJ, Ghali WA. Systematic review and meta-analysis: anti-tumor necrosis factor alpha therapy and cardiovascular events in rheumatoid arthritis. Arthritis Care Res (Hoboken) 2011;63:522-9.
78 Ridker PM, Thuren T, Zalewski A, Libby P. Interleukin-1beta inhibition and the prevention of recurrent cardiovascular events: rationale and design of the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS). Am Heart J 2011;162:597-605.
79 Ridker PM. Testing the inflammatory hypothesis of atherothrombosis: scientific rationale for the cardiovascular inflammation reduction trial (CIRT). J Thromb Haemost 2009;7 Suppl 1:332-9.
80 Manduteanu I, Simionescu M. Inflammation in atherosclerosis: a cause or a result of vascular disorders? J Cell Mol Med 2012;16:1978-90.
81 Anderson TJ, Gerhard MD, Meredith IT, et al. Systemic nature of endothelial dysfunction in atherosclerosis. Am J Cardiol 1995;75:71B-4B.
82 Lobatto ME, Fuster V, Fayad ZA, Mulder WJ. Perspectives and opportunities for nanomedicine in the management of atherosclerosis. Nat Rev Drug Discov 2011;10:835-52.
83 Allen S, Liu YG, Scott E. Engineering nanomaterials to address cell-mediated inflammation in atherosclerosis. Regen Eng Transl Med 2016;2:37-50.
84 Feinberg MW, Moore KJ. MicroRNA Regulation of Atherosclerosis. Circ Res 2016;118:703-20.

8

NEDERLANDSE SAMENVATTING

SAMENVATTING

Stelt u zich eens voor dat u (of een dierbare van u) een hartaanval of beroerte krijgt. U zult dan een gezonde leefstijl nastreven en medicijnen gebruiken om te voorkomen dat dit in de toekomst nog eens gebeurt. Deze aanpak blijkt echter maar matig succesvol: in ruim 2/3 van de gevallen treed er een recidief op. Daarom wordt er wereldwijd uitgebreid onderzoek gedaan naar nieuwe behandelstrategieën om de prognose voor mensen met een sterk verhoogd risico op hart- en vaatziekten te verbeteren. Helaas hebben deze inspanningen tot op heden nog niet geleid tot een belangrijke doorbraak. Wel werd duidelijk dat van alle strategieën gericht op het terugdringen van het risico medicamenteus ingrijpen het meest effectief is. Daarom is het belangrijk om nieuwe geneesmiddelen voor atherosclerose (slagaderverkalking) te ontwikkelen. Gebaseerd op het onderliggende ziekteproces van atherosclerose kunnen deze middelen op verschillende niveaus aangrijpen: 1) het verlagen van het cholesterol, 2) het verminderen van ontsteking, en/of 3) het verbeteren of beperken van de schadelijke gevolgen van vaatwandprikkeling.

VERHOOGD CHOLESTEROL EN ATHEROSCLEROSE

Het hebben van verhoogde waarden van het LDL-Cholesterol (het 'slechte' cholesterol) is de belangrijkste risicofactor voor het ontstaan van atherosclerotische cardiovasculaire ziekte. Daarom wordt in alle internationale behandelrichtlijnen geadviseerd het LDL-C te verlagen bij patiënten die een verhoogd risico hebben op hart- en vaatziekten. Al ruim dertig jaar zijn statines de krachtigste LDL-C verlagende middelen op de markt. Een groot nadeel van deze middelen is dat ze door een aanzienlijk deel van de patiënten niet verdragen worden, of te weinig effectief zijn om de gewenste LDL-C-waarden in het bloed te bereiken, zelfs in hoge doseringen. Daarom is het belangrijk nieuwe LDL-C-verlagende geneesmiddelen te ontwikkelen die goed verdragen worden, en in staat zijn aanzienlijke LDL-C-verlaging te geven. De ontdekking in 2003 dat een bepaald eiwit, PCSK9, een cruciale rol blijkt te spelen in de LDL-C-huishouding, was de lang nagestreefde doorbraak in de zoektocht naar alternatieve methoden voor LDL-C-verlaging. Verhoogde PCSK9-waarden leiden tot verhoogde LDL-C spiegels en bovendien verminderde werkzaamheid van statines, terwijl (aangeboren) lage PCSK9-waarden resulteren in lage LDL-C-waarden en een verlaagd risico op cardiovasculaire ziekte. Deze ontdekking luidde een tijdperk in met een ware hausse aan onderzoeken gericht op het eiwit en het uitschakelen ervan, om zo verhoogde LDL-C-spiegels in het bloed te verlagen.

ONTSTEKING, DE VAATWAND EN ATHEROSCLEROSE

Maar liefst de helft van de patiënten met atherosclerotische cardiovasculaire ziekte heeft geen verhoogde cholesterolwaarden, maar wel verhoogde ontstekingswaarden in het bloed. Er is dan ook overtuigend bewijs geleverd dat bij het ontstaan van atherosclerose en de verdere ontwikkeling ervan diverse ontstekingsreacties betrokken zijn. Belangrijk in dit kader is de Toll-like receptor type 4 (TLR4), gespecialiseerd in het herkennen van patronen die onder meer voorkomen op verschillende ziekteverwekkers, waarvan lipopolysaccharide (LPS, een bestanddeel van een bepaalde soort bacteriën) de bekendste is. Wanneer een dergelijk patroon herkend wordt door TLR4 op ontstekingscellen, worden er processen in gang gezet die leiden tot een ontstekingsreactie, gericht op het onschadelijk maken van de potentiële ziekteverwekker. Behalve lichaamsvreemde stoffen kunnen ook lichaamseigen stoffen TLR4 activeren. Een voorbeeld hiervan is LDL-C: wanneer er in het bloed verhoogde LDL-C-concentraties zijn, dan accumuleert overtollig LDL-C in de vaatwand, waar het aanleiding geeft tot expressie van TLR4 op vaatcellen. Het patroon van het LDL-C wordt vervolgens herkend door de TLR4, resulterend in een ontstekingsreactie. Deze reactie leidt tot beschadiging en functieverlies van het endotheel, de laag cellen die de binnenkant van een bloedvat bekleedt. Hierdoor verliest het endotheel haar fysiologische barrièrefunctie waarbij zij extra doorlaatbaar wordt voor LDL-C-deeltjes vanuit de bloedbaan. Het gevolg hiervan is dat de ontstekingsreactie onderhouden en versterkt wordt, met als gevolg een vicieuze cirkel van buitensporige schade aan de bloedvaten, het fundament voor het ontstaan van atherosclerotische plaques. Er zijn aanwijzingen dat het tegengaan van endotheel dysfunctie atherosclerose vermindert. Dieronderzoek laat bovendien zien dat medicamenteuze remming van TLR4-activiteit resulteert in een afname van atherosclerotische plaques en de mate van ontsteking in het bloed. Daarom is het van groot belang gedetailleerd inzicht te hebben in de ontstekingseffecten en endotheel activatie ten gevolge van TLR4-activatie in mensen, om zo nieuwe geneesmiddelen voor atherosclerose te kunnen ontwikkelen.

ONDERZOEK NAAR GENEESMIDDELEN VOOR ATHEROSCLEROSE IN GEZONDE MENSEN

Er is momenteel geen relevant model voorhanden om in gezonde mensen atherosclerotische ziekteprocessen te onderzoeken, wat de mogelijkheden beperkt om de werkzaamheid van nieuwe medicijnen in gezonde mensen te testen.

Voor het evalueren van middelen met louter cholesterolverlagende werking is dit geen probleem, aangezien verhoogde cholesterolwaarden ook voorkomen in gezonde mensen. Voor het evalueren van de ontstekingsremmende effecten van geneesmiddelen ligt dit anders, omdat gezonde vrijwilligers geen chronische ontsteking hebben. Om in deze populatie toch ontstekingsremmende medicijnen te kunnen testen is daarom een ziektemodel vereist, waarin een ontstekingssituatie nagebootst wordt. In het endotoxemiemodel wordt bij gezonde vrijwilligers LPS in de bloedbaan gespoten om zo een TLR4-gedreven ontstekingsreactie te genereren, die in het bloed gemeten kan worden. De ruime ervaring met dit model is hoofdzakelijk gebaseerd op studies waarin relatief hoge doseringen LPS (4 ng/kg) werden toegediend, die klinische effecten opwekken vergelijkbaar met een ernstige ontsteking, zoals sepsis (bloedvergiftiging). Dergelijke hoge doseringen zijn belastend voor vrijwilligers, en veroorzaken een hevige ontstekingsreactie. Literatuur suggereert dat milde endotoxemie in gezonde vrijwilligers een ontstekingsreactie in het bloed teweeg brengt die lijkt op de ontsteking aanwezig in het bloed van patiënten met atherosclerose. Bovendien zijn er aanwijzingen dat relatief hoge doseringen LPS (meer dan 2 ng/kg) toegediend aan gezonde vrijwilligers endotheelactivatie kan opwekken, zoals ook bij atherosclerose gezien wordt. Over de dosis-effectrelatie van lage doseringen LPS (<2 ng/kg) op ontsteking en endotheel activatie is echter weinig bekend. Kennis hierover is belangrijk, omdat deze als basis kan dienen voor de ontwikkeling van geneesmiddelen gericht op verlaging van de ontstekingscomponent van atherosclerose.

Samengevat is er behoefte aan nieuwe geneesmiddelen die het (herhaald) optreden van hart- en vaatziekten vermindert. Potentiële strategieën hiervoor zijn het verlagen van het LDL-Cholesterol via PCSK9-remming, het verminderen van TLR4-gemedieerde ontsteking, en het verminderen van vaatwandactivatie. In dit proefschrift worden de resultaten beschreven van een tweetal onderzoeken naar de mogelijke toepasbaarheid in mensen van een nieuwe PCSK9-remmer en een nieuw TLR4-blokkerend geneesmiddel. Ook werd onderzocht of de laaggradige endotoxemietest als humaan model gebruikt kan worden voor de inflammatoire component van atherosclerose, om zo de werkzaamheid van toekomstige geneesmiddelen voor atherosclerose in een vroeg klinisch stadium te kunnen testen.

DIT PROEFSCHRIFT

In **hoofdstuk 2** van dit proefschrift beschrijven we de resultaten van een onderzoek waarin we een nieuw PCSK9-verlagend middel voor het eerst aan gezonde proefpersonen met een verhoogd LDL-C hebben toegediend. Al in relatief lage hoeveelheden (tot 5 mg/kg) verlaagde dit middel het PCSK9 met 50%, en het LDL-C in het bloed met 25%. Vanwege schadelijke effecten op de nieren en op de plaatsen onder de huid waar het middel was toegediend (injectieplaatsreacties) moest dit onderzoek voortijdig worden gestopt. Met name de effecten op de nier waren zorgwekkend, omdat de nieren essentiële lichaamsfuncties vervullen, en omdat dergelijke schadelijke effecten onverwacht waren. Middelen met vergelijkbare chemische structuur (antisense oligonucleotides) waren niet eerder in verband gebracht met negatieve effecten op de nierfunctie, zelfs niet in veel hogere doseringen. In geen van de voorgaande preklinische onderzoeken met het middel zelf (een locked nucleic acid, een subklasse van de antisense oligonucleotides) waren er aanwijzingen geweest dat het middel schadelijk voor de nier zou kunnen zijn. Ook was nooit melding gemaakt van een mogelijk verband tussen lage PCSK9-spiegels en het optreden van nierfunctiestoornissen. Wij hebben uitvoerig onderzocht waarom het middel in ons onderzoek wel schadelijk bleek te zijn voor de nier, en hebben de resultaten beschreven in **hoofdstuk 3**.

In de kliniek is de gouden standaard voor het vaststellen van nierfunctiestoornissen het aantonen van een stijging van bepaalde afvalstoffen in het bloed (creatinine, ureum). Echter, dit schadesignaal openbaart zich pas ongeveer 3 dagen nadat de nier daadwerkelijk beschadigd werd. De opgetreden nierschade kan dan al aanzienlijk zijn. Bovendien vertellen deze afvalstoffen niet wat de precieze oorzaak van de schade is, en welke onderdelen van de nier zijn aangedaan (bijvoorbeeld de nierbuisjes, de tubuli, of de haarvatenkluwen, de glomerulus). De laatste jaren verschijnen er steeds meer onderzoeken over stoffen in de urine die eerder laaggradige schade (prikkeling) zouden kunnen weerspiegelen, en bovendien informatie geven over de locatie van de schade. Om deze reden hebben we een aantal van dergelijke schademarkers gemeten. We toonden aan dat een verhoging van enkele van deze stoffen in de urine voorspellend kan zijn voor het pas dagen later optreden van nierfunctiestoornissen in het bloed, en dat het eerste deel van de nierbuisjes aangedaan was. Onze publicatie, waarin wij waarschuwen voor de potentieel nadelige effecten op de nier van deze subgroep geneesmiddelen (de locked nucleic acids), heeft geleid tot stringentere controle van de nierfunctie in studies met deze groep geneesmiddelen. Dat het middel dat wij in onze studie toedienden te schadelijk was om verdere ontwikkeling ervan te rechtvaardigen, is teleurstellend, aangezien het steeds duidelijker wordt dat de strategie van PCSK9-verlaging op zichzelf de lang nagestreefde doorbraak zou kunnen zijn in de behandeling van verhoogd LDL-C. Verschillende monoclonale antilichamen gericht tegen PCSK9 blijken niet alleen veilig te zijn, maar geven bovendien niet eerder geëvenaarde LDL-C-verlaging van 50-70%, en daling van het risico op hart- en vaatziekten.

Behalve via LDL-C-verlaging kan het risico op hart- en vaatziekten verlaagd worden door het remmen van laaggradige ontsteking. In **hoofdstuk 4** tonen wij aan dat laaggradige ontsteking nagebootst kan worden door gebruik te maken van het humane endotoxemiemodel: het simpelweg toedienen van een lage dosis (0.5, 1 of 2 ng/kg) LPS blijkt een veilige manier om in het bloed van gezonde vrijwilligers een kortdurende en milde ontstekingsreactie teweeg te brengen. We evalueerden niet alleen de ontstekingsreactie in het bloed van de proefpersonen (*in vivo*) die LPS hadden gekregen, maar ook de ontstekingsreactie in een bloedbuisje (*ex vivo*) van de vrijwilligers waaraan we LPS toedienden in het laboratorium. Hoewel de beide LPS-testen natuurlijk wezenlijk verschillen van elkaar, bleek het opgewekte ontstekingseffect tussen beide vergelijkbaar. Hiermee tonen we aan dat de *ex vivo* LPS test een niet-invasief alternatief kan zijn voor de *in vivo* LPS-toediening in vrijwilligers. Onze nauwkeurige beschrijving van de ontstekingseffecten (*in vivo* en *ex vivo*) over de tijd die gezien worden na toediening van deze verschillende doses LPS kan gebruikt worden voor het ontwerpen van toekomstige studies met nieuwe laaggradige ontstekingsremmers. Een belangrijk praktisch aspect om rekening mee te houden hierin is 'LPS hyporesponsiveness', het fenomeen dat de *ex vivo* ontstekingsreactie tijdelijk geremd is na eerdere *in vivo* LPS-toediening. In de literatuur was beschreven dat dit fenomeen binnen een week verdwenen was, een grove schatting van de duur van de hyporesponsiveness die in de praktijk niet erg bruikbaar is voor gecombineerde *in vivo*/*ex vivo* methodiek. Wij lieten zien dat deze remming maximaal was 6 uur na LPS-toediening en al binnen 12 uur verdwenen is. Vervolgens hebben we dit LPS-model toegepast in een klinisch onderzoek naar de werkzaamheid van een nieuw middel, een monoclonaal antilichaam, ontwikkeld om de TLR4-gedreven ontstekingsreactie te remmen (**hoofdstuk 5**). In het eerste deel van het onderzoek dienden we aan gezonde vrijwilligers een enkele dosis van het middel toe en testten we de remming van de ontstekingsreactie in het bloed van proefpersonen, zowel voorafgaand als herhaaldelijk na het toedienen van het middel. Het middel werd goed verdragen in een dosis variërend van 0.001 mg/kg tot 15 mg/kg, was erg potent (al vanaf 1 mg/kg bijna volledige remming van de LPS-geïnduceerde ontstekingsreactie), en werkte langdurig (ongeveer 10 weken bij de hoogste dosering). Een verhoogde infectiegevoeligheid werd niet geobserveerd, waarschijnlijk omdat adequate afweer tegen ziekteverwekkers geborgd wordt door TLR4-onafhankelijke processen. In dit deel van het onderzoek brachten we het ontstekingsremmend effect van oplopende geneesmiddelconcentraties in kaart, tot een concentratie die theoretisch nodig zou zijn om op weefselniveau werkzaam te zijn. In het tweede deel van het onderzoek onderzochten wij of het middel ook daadwerkelijk klinisch in staat was om de LPS-gemedieerde ontstekingsreactie (snelle hartslag, griepachtige verschijnselen, en temperatuurverhoging) te remmen. Dit evalueerden wij op drie tijdstippen na de toediening van het geneesmiddel, namelijk direct, of na 22 of 40 dagen. We toonden aan dat het experimentele middel al in een lage dosis deze verschijnselen kon voorkomen, en dat dit effect gedurende minimaal 3 weken aanhield. De mate en duur van het effect van het antilichaam was goed vergelijkbaar wanneer het werd vastgesteld aan de hand van een test *in vivo*, en een test *ex vivo*. Dit inzicht is belangrijk aangezien het ontstekingsmodel *in vivo* belastend is voor de vrijwilliger, en bovendien niet frequent herhaald kan worden over de tijd, in tegenstelling tot ontstekingstesten *ex vivo*.

In bovenstaand onderzoek hebben we de LPS-stimulatie *in vivo* toegepast om de werkzaamheid van een TLR4-blokker te bestuderen, toegespitst op de ontstekingsreactie. Het effect van LPS op het menselijk lichaam is echter veel breder. Endotoxemie kan niet alleen ontstekingscellen aanzetten tot het uitstoten van ontstekingsmediatoren, maar ook de vaatwand activeren, en misschien zelfs in ernstige gevallen leiden tot nierschade. Gedetailleerde kennis over de relatie tussen LPS-dosis en effect op vaatwand en nier ontbreekt echter. Ook bij atherosclerose wordt endotheelactivatie gezien. De endotoxemietest zou daarom mogelijk ook gebruikt kunnen worden als atherosclerosemodel, om de effectiviteit te testen van nieuwe middelen die de vaatwand moeten beschermen tegen ontstekingsprikkels. De toepasbaarheid van experimentele endotoxemie als model voor endotheelactivatie in gezonde vrijwilligers hebben we onderzocht in **hoofdstuk 6**. Dat LPS-toediening aan gezonde vrijwilligers in relatief hoge doses (2 en 4 ng/kg) leidt tot endotheelactivatie was eerder beschreven, maar systematisch verzamelde informatie met betrekking tot dosis-effectrelaties bij toediening van lagere doses LPS was niet voorhanden. Wij toonden robuuste, dosisafhankelijke endotheelactivatie na toediening van lage doses LPS. Dit is relevante informatie omdat het laaggradige endotoxemiemodel dat wij toepasten hiermee geschikt zou kunnen zijn voor toekomstige onderzoeken met geneesmiddelen gericht op het beschermen van de integriteit van het endotheel.

Veiligheid voor de gezonde vrijwilliger van interventies in onderzoeksverband is vanzelfsprekend van groot belang. Eerdere onderzoeken toonden dat toediening van relatief hoge hoeveelheden LPS (hoger dan 2 ng/kg lichaamsgewicht) aan gezonde vrijwilligers kan resulteren in plotselinge nierschade gemeten met nieuwe en gevoelige urinemarkers. Wij hebben een uitgebreid arsenaal van dergelijke niermarkers gemeten en vonden dat 0.5 en 1 ng/kg LPS geen effect hierop had. Bij de hoogste dosering LPS (2 ng/kg) waren er aanwijzingen dat LPS mogelijk effect op de nierbuisjes begon te krijgen, overigens zonder functionele consequenties. Omdat dit

effect kortstondig was, slechts een halve dag, er geen nierfunctiestoornissen optraden, en de markers bovendien slechts marginaal verhoogd waren vergeleken met patiënten met nierschade, concludeerden wij dat het toedienen van lage doses LPS in experimentele context geen nadelige gevolgen heeft op de nieren van gezonde proefpersonen, en veilig gebruikt kan worden als ziektemodel in geneesmiddelonderzoek.

CONCLUSIE

De onderzoeken beschreven in dit proefschrift zijn relevant voor toekomstige medicamenteuze interventies gericht op de belangrijkste pathofysiologische aspecten van atherosclerose, namelijk 1) te hoge bloedwaarden van het LDL-C, 2) laaggradige ontsteking, en 3) dysfunctionerend endotheel.

Ten aanzien van het eerste aspect toonden wij dat LDL-C in het bloed effectief verlaagd kan worden door toediening van een PCSK9-remmend 'locked nucleic acid'. Helaas moesten we ook vaststellen dat dit geneesmiddel (ernstige) achteruitgang van de nierfunctie gaf ten gevolge van schade aan de nierbuisjes. Met behulp van nieuwe, gevoelige schademarkers lieten wij zien dat dergelijke schade mogelijk dagen eerder opgespoord kan worden dan met conventionele bloed- en urinetesten. Bovendien leidde de experimentele behandeling tot ongewenste reacties rondom de injectieplaats. Deze bevindingen demonstreren dat effecten van dit PCSK9-remmende middel, of mogelijk van deze klasse geneesmiddelen, in primaten in preklinische toxicologie studies niet betrouwbaar bijwerkingen op de nieren of de huid in mensen voorspellen.

Verder presenteren wij dat laaggradige endotoxemie, geïnduceerd door toediening van een lage dosis LPS (≤2 ng/kg), een milde ontstekingsreactie en endotheelactivatie teweeg brengt in gezonde vrijwilligers, en dat dit niet gepaard gaat met schadelijke effecten op de nier. Gebruik makend van deze methodologie toonden wij dat TLR4-gemedieerde ontstekingseffecten met een experimenteel monoclonaal antilichaam gericht tegen TLR4 volledig en langdurig onderdrukt konden worden, zonder indicaties voor een verhoogde infectiegevoeligheid. Vanuit klinisch-farmacologisch oogpunt is dit onderzoek interessant omdat het illustreert hoe op basis van basale moleculaire-celbiologische kennis een relatief simpele bloed-gebaseerde ontstekingstest kon worden toegepast die in gezonde vrijwilligers het gewenste effect van het middel aantoonde. Dit maakte een rationele en veilige onderzoeksopzet mogelijk, omdat de test zowel beschouwd kan worden als farmacodynamische test, maar ook als veiligheidstest: de test verschafte immers een directe kwantitatieve maat voor onderdrukking van het aangeboren immuunsysteem. Op basis van een gecombineerd farmacokinetisch en farmacodynamisch model werd het mogelijk om efficiënt de optimale toe te dienen doses van het experimentele middel te voorspellen, en het gewenste tijdsinterval tussen de toedieningen. We bevestigden de validiteit van de bloed-gebaseerde ontstekingstest door in een klein aantal vrijwilligers ook een ontstekingsreactie op te wekken *in vivo*, door middel van de eerder genoemde endotoxemietest. Hiermee toonden we de betrouwbaarheid van het geschatte effect van het experimentele middel op basis van de bloed-gebaseerde ontstekingstest. Deze door ons gegenereerde informatie kan als basis dienen voor de verdere ontwikkeling van dit experimentele middel, theoretisch voor iedere pathologische conditie met een belangrijke TLR4-component.

Tenslotte lieten wij zien dat het laaggradige endotoxemiemodel ook gebruikt kan worden om endotheelactivatie te induceren. Aangezien dit een belangrijk pathofysiologisch aspect is van atherosclerose onderstreept dit de relevantie van het model voor toekomstig klinisch-farmacologisch onderzoek met experimentele anti-atherosclerotische middelen, bijvoorbeeld bedoeld om het endotheel te beschermen.

BIBLIOGRAPHY

EP van Poelgeest, MR Dillingh, KE Malone, EM Kemper, ESG Stroes, J Burggraaf, M Moerland. Characterization of immune cell, endothelial, and renal responses upon human low-dose experimental endotoxemia. *Journal of Pharmacological and Toxicological Methods 2018; 89: 39-46.*

E Monnet, G Lapeyre*, **EP van Poelgeest***, P Jacqmin, K de Graaf, J Reijers, M Moerland, J Burggraaf, C de Min. Evidence of NI-0101 pharmacological activity, an anti-TLR4 antibody, in a randomized phase I dose escalation study in healthy volunteers receiving LPS. *Clinical Pharmacology and Therapeutics 2017 Feb;101(2):200-208. (*Equal contribution)*

EP van Poelgeest, M Moerland, M Hodges, Y Tessier, AC Cohen, J Burggraaf. Antisense-mediated reduction of Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9): a first-in-human randomized, placebo-controlled trial. *British Journal of Clinical Pharmacology 2015 Dec;80(6):1350-61.*

MR Dillingh*, **EP van Poelgeest***, KE Malone, EM Kemper, ESG Stroes, M Moerland. Characterization of inflammation and immune cell modulation induced by low-dose LPS administration to healthy volunteers. *Journal of Inflammation 2014, 11:28. (*Equal contribution)*

EP van Poelgeest, RM Swart, MG Betjes, M Moerland, JJ Weening, Y Tessier, MR Hodges, AA Levin, J Burggraaf. Acute kidney injury during therapy with an antisense oligonucleotide directed against PCSK9. *American Journal of Kidney Disease 2013 Oct;62(4):796-800.*

JJ Westenberg, **EP van Poelgeest**, P Steendijk, H Grotenhuis, WJ Jukema, A de Roos. Bramwell-Hill Modeling for local aortic pulse wave velocity estimation: a validation study with velocity-encoded MRI and invasive pressure assessment. *Journal of Cardiovascular Magnetic Resonance 2012 Jan 9;14:2.*

EP van Poelgeest, HJ Baelde, EL Lagaaij, YW Sijpkens, JA Bruijn, IM Bajema. Endothelial cell chimerism occurs more often and earlier in female than in male recipients of kidney transplants. *Kidney International 2005 Aug;68(2):847-53.*

EP van Poelgeest, HC van Houwelingen, E de Heer, EL Lagaaij, JA Bruijn, IM Bajema. Micro- and tissue chimerism in physiologic, autoimmune, and transplantation settings: a meta-analysis. *Transplantation Reviews 2004, 18 (3), 153-161.*

CURRICULUM VITÆ

Eveline Petra van Poelgeest was born on October 2, 1978 in Almelo. She completed secondary education in 1997 (Gymnasium Beta, O.S.G. De Bataafse Kamp, Hengelo, The Netherlands). She studied Law at the Leiden University from 1997 to 1999. She studied Medicine at the Leiden University Medical Center, from which she graduated as Doctor of Medicine in 2003. After two years of internship she obtained her qualification as a Medical Doctor (*cum laude*). She started her professional carrier as a resident in Internal Medicine at the Rode Kruis hospital in The Hague and Leiden University Medical Center. The last two years of her training she dedicated to the subspecialty Vascular Medicine under supervision of Prof. Dr. M.V. (Menno) Huisman, Prof. Dr. S. (Saskia) Middeldorp and Dr. J. (Jouke) Tamsma. During her fellowship, she started her PhD trajectory as described in this thesis at the Center of Human Drug Research under supervision of Prof. dr. J. (Koos) Burggraaf, Prof. dr. A.F. (Adam) Cohen and Dr. M. (Matthijs) Moerland. As part of her research training, she became a board-certified Clinical Pharmacologist in 2013. In 2014/2015 she worked as a staff member at the department of Pharmacology, Vascular and Metabolic Medicine of the Erasmus Medical Center in Rotterdam, and subsequently as a staff member at the geriatric department in the Alrijne hospital (Leiderdorp and Leiden). Since July, 2017, she works at the geriatric department of the Academic Medical Center in Amsterdam, where she is in training to become an internist specialized in care for the elderly. She lives in Leiden with Kim Groothuis and their children Meike and Lucas.