Future drugs in atherosclerotic cardiovascular disease

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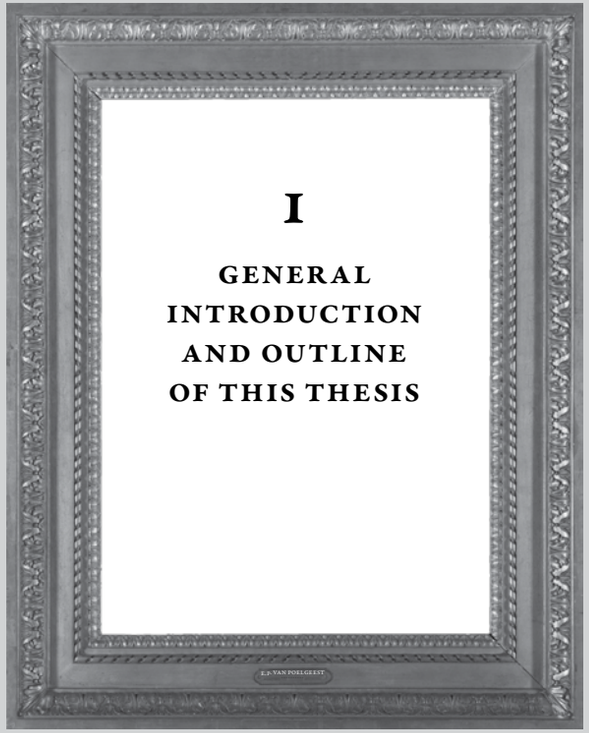


I

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).

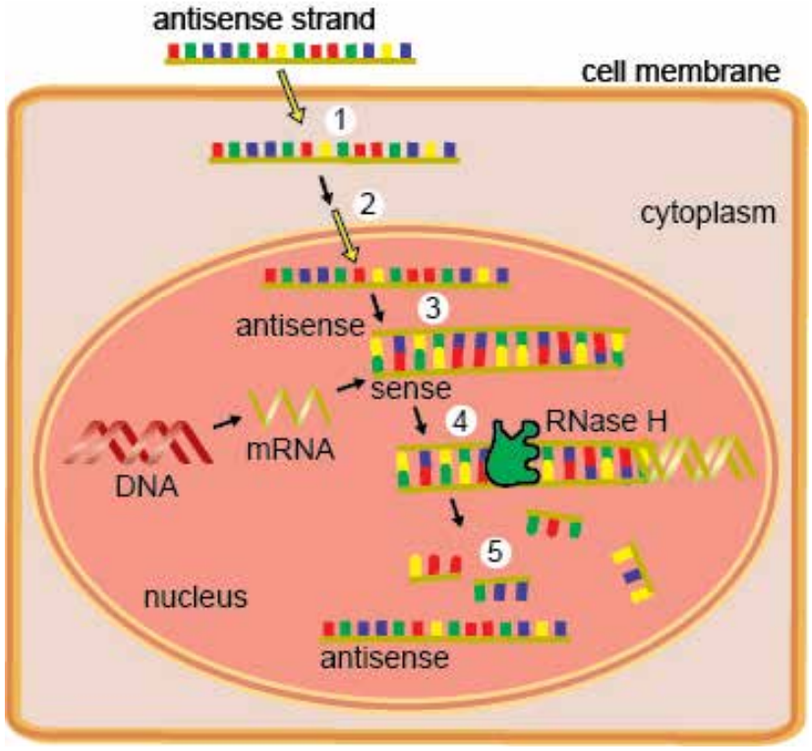
The results of our TLR4 challenge test guided the design of our clinical trial described in **CHAPTER 5**, in which we explored the anti-inflammatory potential of NI-0101 in *in vivo* and *ex vivo* LPS challenge tests (Figure 3C).

TARGETING VASCULAR DYSFUNCTION

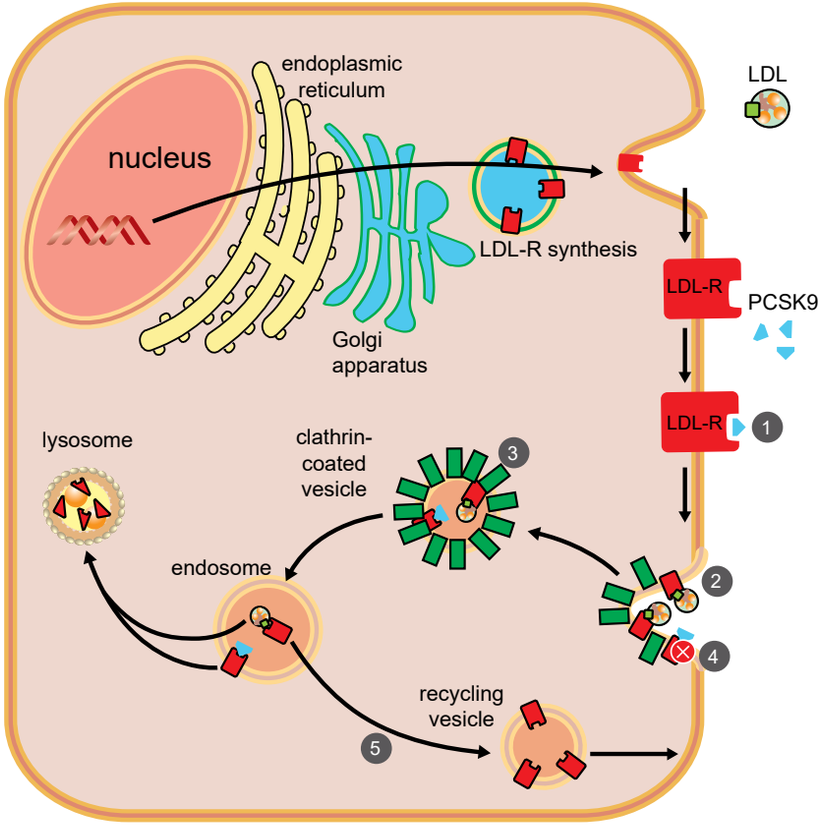
Pharmacological interventions targeting endothelial activation/dysfunction may be an interesting approach in ACVD because it links hypercholesterolemia and inflammation, two key players in the pathophysiology of atherosclerotic disease. Healthy endothelial cells effectively maintain vascular wall homeostasis. 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

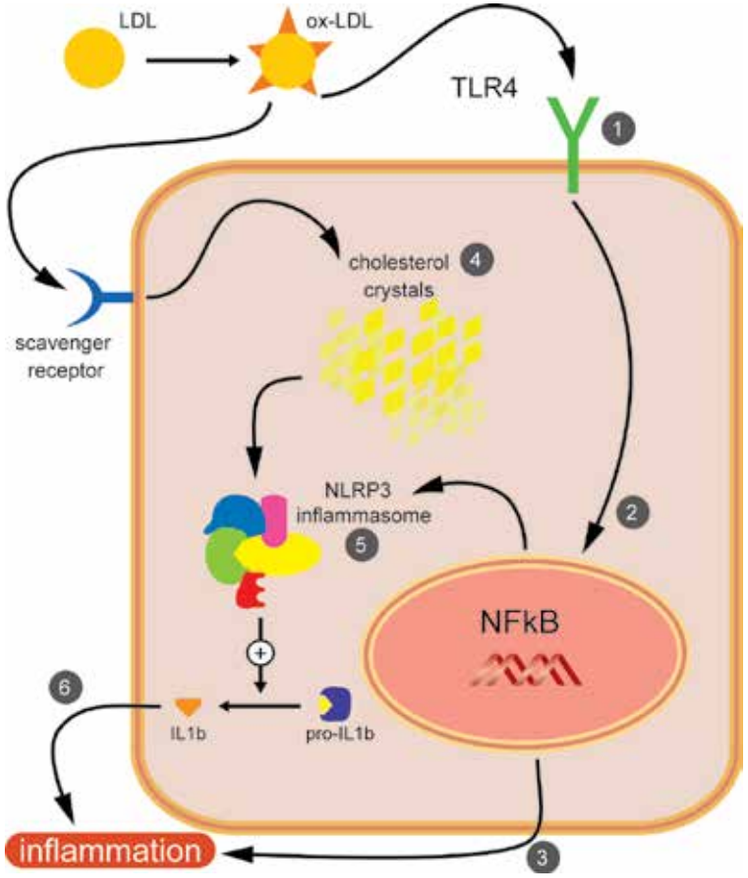


PANEL A. ANTISENSE OLIGONUCLEOTIDE MECHANISM OF ACTION. Single-stranded oligonucleotides are transported across the plasma membrane (step 1). In the cytoplasm, single-stranded oligonucleotides rapidly accumulate in the nucleus (steps 2 and 3), where they bind to their targeted RNA (step 4). Once bound 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.



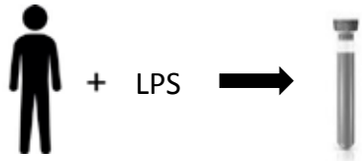
PANEL B. PCSK9 MECHANISM OF ACTION, AND PCSK9 INHIBITION BY SPC5001. PCSK9 binds to the LDL-R, locking it to an open configuration (step 1); the resulting complex is transported from the cell membrane into the cell by clathrin-mediated endocytosis (step 2); the open configuration of the LDL-R directs 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.

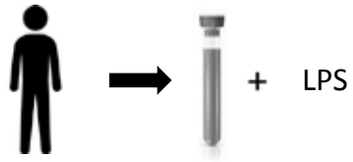


Both exogenous (e.g. lipopolysaccharides) and endogenous (e.g. oxidized LDL-C) ligands can ligate TLR4 (step 1) on cells such as macrophages, vascular smooth muscle cells, dendritic cells and endothelial cells. Ligand binding activates the myeloid differentiation primary response protein 88 (MYD88)-dependent 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).

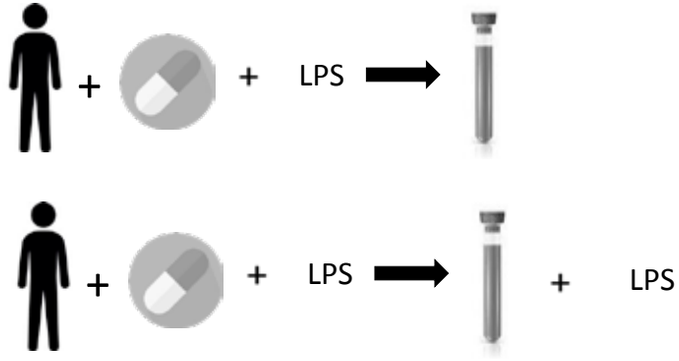
Figure 3. Schematic representation of the methodological and pharmacological interventions applied in this thesis.



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).

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