Cholesterol metabolism in mouse models of atherosclerosis and adrenal steroidogenesis

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GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Cholesterol is a vital component in cellular metabolism as well as the building block for steroidogenesis, including the production of adrenal-derived glucocorticoids. Misbalance in the concentration of cholesterol has been linked to a variety of diseases and pathologies, among which are cardiovascular disease (CVD) and metabolic syndrome [1]. The exact interplay between cholesterol levels, glucocorticoids and atherosclerosis is still under debate. In this thesis we discuss the role of cholesterol, specifically high-density lipoprotein cholesterol (HDL-C), during the progression and regression of atherosclerosis. In addition, the effect of intracellular and extracellular cholesterol on adrenal steroidogenesis of glucocorticoids is described.

HDL CHOLESTEROL AND ATHEROSCLEROSIS

CVD events like myocardial infarction, ischemic stroke and co-morbidities including the metabolic syndrome are the leading cause of death in the Western society. The driving pathology behind CVD is atherosclerosis. Atherosclerosis is initiated by a complex interplay between relatively high cholesterol levels and inflammatory components. It is generally accepted that lowering the levels of the low-density lipoprotein cholesterol (LDL-C) fraction reduces the risk for a CVD event. Current interventions strategies achieve this goal mainly via treatment with statins, HMG-CoA reductase inhibitors. However, statin-induced cholesterol reduction lowers the risk of CVD mortality by only approximately 25 % [2], indicating that there is a high need for additional therapies to diminish the CVD burden. Epidemiological studies showed a strong association between low levels of HDL-C and risk for a CVD event. It was therefore hypothesized that increasing HDL might be a valuable strategy to further reduce CVD related mortality and halt progression or even induce regression of atherosclerosis [3-5]. In line, genetically modified animal models revealed that the presence of HDL is of great importance for the initiation of lesion regression [6]. In mice, increased concentrations of HDL, via human ApoA1 overexpression [7,8] or infusion of ApoA1milano, halted progression and initiated atherosclerotic lesion regression. The relationship between HDL and reverse cholesterol transport (RCT) led to the development of HDL increasing drugs and subsequent clinical trials to manipulate lesion development. However, the outcome of these trials was disappointing, showing that increased levels of HDL-C on top of a statin treatment did not stimulate regression of existing lesions [9-11]. The importance of HDL-C quantity was further questioned in humans and mice with a genetically variation in SR-BI, a key receptor in hepatic uptake and metabolism

of HDL cholesteryl esters. SR-BI deficiency results in an augmented level of HDL-C and abnormal enlargement of HDL particles, leading to an increased rather than a decreased susceptibility for atherosclerosis [12]. These observations combined marked a shift in the general discussion around HDL and its anti-atherogenic properties from the importance of HDL-C quantity towards the significance of the functionality of a HDL particle [13,14].

A key modulator of HDL is the phospholipid transfer protein (PLTP) regulating the level, size and composition. PLTP is involved in the transfer of phospholipids from VLDL towards pre-β HDL and α-HDL during lipolysis [15]. Total-body deletion of PLTP in mice led to decreased HDL-C and phospholipid levels and an increase in the non-HDL phospholipid fraction upon a high fat challenge [16].

By additional deletion of PLTP in SR-BI knockout (KO) mice, we provoked normalization of the HDL particle size and reduced HDL-C accumulation (**Chapter 2**). In addition to the lowered amount of cholesterol transported in the HDL fraction, a reduction in atherosclerosis susceptibility was recorded. This finding again stressed that HDL-C quantity is not necessarily predictive for atherosclerotic susceptibility. Under normal conditions, PLTP is also expressed by CD68-positive macrophages in atherosclerotic plaques [19]. Furthermore, in vitro cultured macrophages loaded with acetylated-LDL displayed a paralleled increase in PLTP expression [19]. Our group showed that the absence of macrophage-derived PLTP lowers blood cholesterol levels and decreases the atherosclerotic susceptibility in LDLr KO mice on Western-type diet (WTD) [20]. These data combined suggest that deletion of PLTP locally in macrophages of the atherosclerotic plaque could be of direct influence on macrophage foam cell formation and thus atherogenesis. However, it is most likely that the reduced atherosclerosis susceptibility of PLTP/SR-BI double knockout mice is the result of restored HDL functionality.

The key measure of HDL functionality is defined by its cholesterol efflux capacity from macrophages, which by itself has a strong inverse association with carotid intima thickness independently of HDL-C levels [17,18]. Normalization of HDL-particle size in SR-BI KO mice via the additional deletion of PLTP indeed led to the formation of particles that were more effective in stimulating macrophage cholesterol efflux. In recent years the understanding of HDL metabolism and functionality has improved largely. However, considering that HDL comprises a heterogeneous population of particles with a wide variety of proteins in its shell that influence its function, strategies

aimed at increasing a specific function of the HDL particle without impairing another will remain a challenge. Therefore, it might be more promising to target macrophages in atherosclerotic plaques to stimulate macrophage cholesterol efflux and augment reverse cholesterol transport.

Macrophage cholesterol efflux is mainly established via the ATP-Binding Cassette (ABC) transporter subfamily A1 and G1 (ABCA1 and ABCG1) promoting cholesterol transport towards lipid-poor apolipoprotein A1 (ApoA1) or mature HDL particles, respectively (**Figure 1**)[21-25].

The major regulator of these ABC transporters is the Liver X receptor (LXR). Upregulation of ABCA1 and ABCG1 by LXR agonists stimulates cholesterol efflux from macrophages in vitro. In addition, treatment with LXR agonists results in the disappearance of macrophages from atherosclerotic lesions leading to plaque regression in mice [26-28]. This outcome suggested that LXR could serve as an attractive therapeutic target to reduce CVD incidence. However, LXR's have a more broad role in lipid metabolism. This includes the regulation of hepatic lipogenesis. Administration of synthetic LXR agonists to reduce the CVD incidence in mice increased the levels of plasma triglycerides and very low-density lipoproteins (VLDL) as well as hepatic steatosis in response to LXR agonist treatment [29]. Considering these unwanted systemic effects of LXR agonism, it will be interesting to follow the generation of novel LXR related therapeutic intervention strategies that circumvent the known obstacles and specifically improve macrophage cholesterol efflux capacity, e.g. by nanoparticle-mediated selective delivery of LXR agonist to macrophages [30, 31], to halt the progression and initiate regression of lesions.

Although animal models proved that regression of existing lesions is feasible, the underlying mechanisms remain under debate. Mouse models of lesion regression, either by controlled inactivation of the hyperlipidemia (Reversa mouse) [32] or by transplantation of a lesion containing aortic segment into a normolipidemic mouse (aorta transplantation) [33], suggested that macrophage emigration from the lesion site drives atherosclerosis regression under extreme lipid lowering conditions. Besides lowering cholesterol levels, Rahman et al. showed that for lesion regression a continuous recruitment of Ly6C^{hi} monocytes is required, suggesting a dynamic role for the Ly6C^{hi} monocytes in the lesion [34]. Alternatives to the immigration/emigration hypothesis have been provided by studies executed in ApoE knockout mice with a reversed hypercholesterolemia induced by restoration of ApoE via viral transfection.

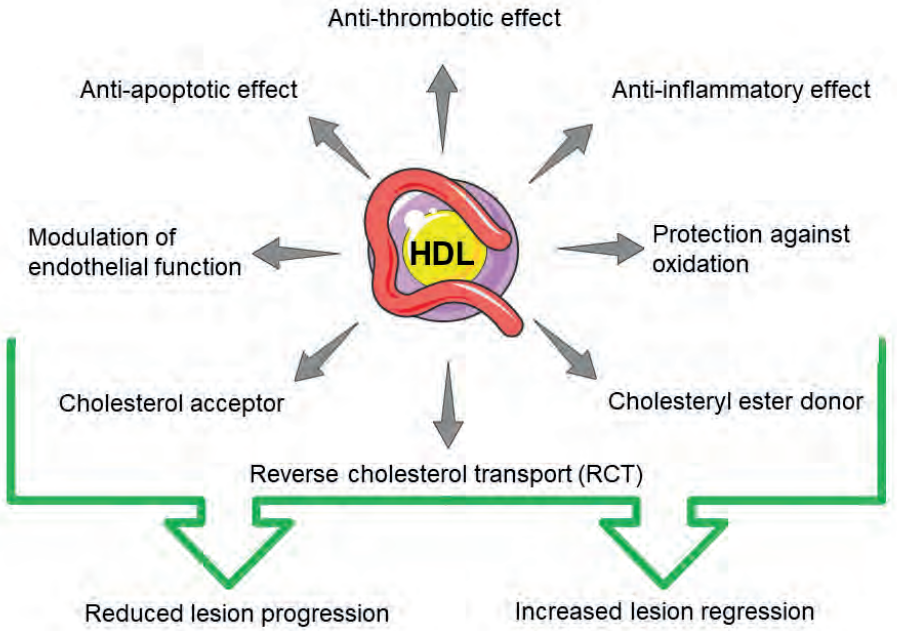

Figure 1: Summary of the anti-atherogenic properties of HDL leading to reduced lesion progression and increased lesion regression.

These studies indicate that regression of existing lesions is facilitated by a combination of impaired recruitment of monocytes and apoptosis of lesional macrophages [35]. The different conclusions of the mechanisms underlying lesion regression could theoretically be dependent on the animal model used. The animal models used are based on lowering the non-HDL fraction while maintaining HDL levels as vehicle for the reuptake of excess cholesterol from the lesions. Our group has recently developed an additional atherosclerosis regression model with the aim to provide more insight in the regression process. More specifically, Van der Stoep et al. showed that reintroduction of bone marrow-derived ApoE in ApoE knockout mice results in a normalization of the hypercholesterolemia and subsequently regression of existing lesions [28]. In **Chapter 3** we tested the hypothesis that the presence of HDL is necessary to initiate lesion regression in this experimental setup. We showed that probucol-induced HDL deficiency impairs the ability of established lesions to regress in response to reversal of the genetic hypercholesterolemia in ApoE knockout mice by reintroduction of ApoE-producing bone marrow-derived cells. Thus independent of the model used, functional HDL is important for stimulating atherosclerosis regression (**Figure 1**).

CHOLESTEROL AND GLUCOCORTICOIDS

In steroidogenesis, cholesterol serves as the sole substrate for the production of steroid hormones including adrenal-derived cortisol in humans and corticosteroids in rodents. However, the contribution of individual lipoprotein species to the generation of the adrenal cholesterol pool remains unknown. In humans, both LDL-C and HDL-C deficiency results in glucocorticoid insufficiency [36,37] suggesting that both LDL and HDL are contributing to the adrenal cholesterol pool. Where the non-HDL fraction is the dominant lipoprotein fraction in humans, the principal lipoprotein fraction in mice is HDL. In **Chapter 4** of this thesis we showed that any alterations to the HDL fraction in murine models results in a reduction of the adrenal cholesteryl ester stores and subsequently leading to a glucocorticoid insufficiency. Also, mice with a human-like lipoprotein profile, transporting the majority of cholesterol in blood by LDL and relatively little by the HDL fraction, display a depletion of the adrenal cholesteryl ester stores [38]. Interestingly, ApoA1 x LDL receptor double knockout mice, lacking the HDL fraction while transporting large amounts of cholesterol in the non-HDL fraction, exhibited a normal plasma corticosterone response despite depletion of the adrenal cholesteryl ester stores [39]. Moreover, in ApoE knockout mice the non-HDL fraction driven hyperlipidemia is paralleled by hypercorticosteronemia and normal cholesteryl ester-stores. These observations let us hypothesize that in mice adrenal cholesteryl ester pools are not the primary source for steroidogenesis and that the loss of HDL can be compensated by the use of cholesterol from non-HDL fractions for steroidogenesis. In **Chapter 4** we examined the impact of the specific lowering of non-HDL levels on adrenal cholesterol and glucocorticoid homeostasis in ApoE knockout mice through transplantation with ApoE-containing wild-type bone marrow. Interestingly, reversal of the hyperlipidemia in ApoE knockout mice was associated with a decreased adrenal glucocorticoid output and accompanied by an unchanged adrenal cholesteryl ester pool. Our studies suggest that in mice at least two cholesterol mobilization routes can be discriminated within the adrenals to maintain a high rate of steroidogenesis (see **Figure 2**): 1) a route in which HDL provides cholesterol to the adrenals for cholesteryl ester storage and that can be subsequently be used for steroidogenesis and cellular metabolism and 2) a route in which cholesterol taken up via the VLDL/LDL (non-HDL fraction) is directly utilized for adrenal steroidogenesis.

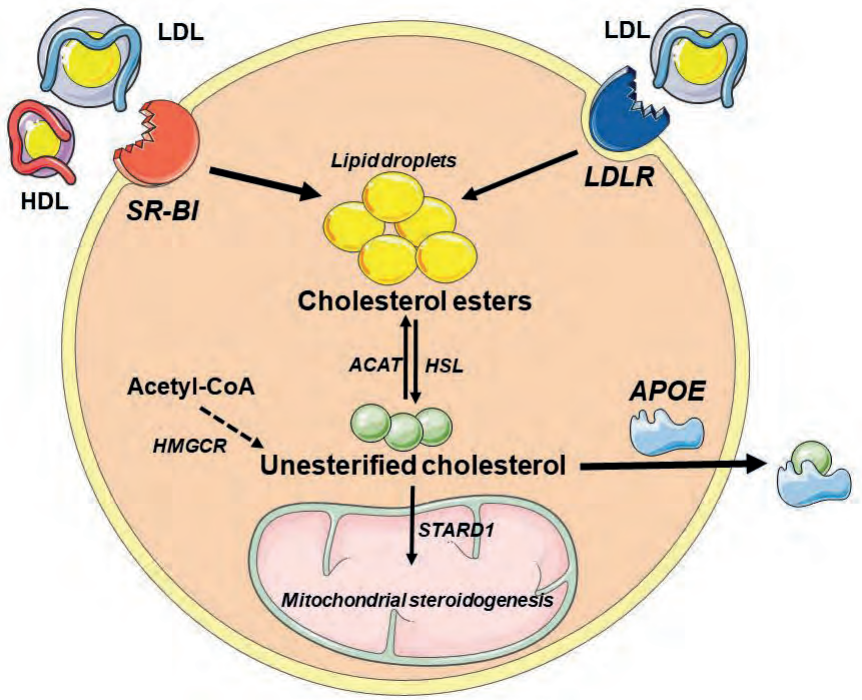

Figure 2: Schematic overview of an adrenal cell with the various routes and sources that provide cholesterol for adrenal steroidogenesis and the suggested role for Apolipoprotein E (ApoE) in the efflux of excess cholesterol from the lipid stores.

The role of the different receptors involved in adrenal cholesterol metabolism is still under debate. The three major lipoprotein receptors available for cholesterol uptake in the adrenal are the LDL receptor, low-density lipoprotein receptor-related protein 1 (LRP1), and SR-BI. We previously demonstrated, via adrenal transplantation, that the LDL receptor is not contributing to the overall adrenal cholesteryl ester pool [40]. Functional deficiency for LRP1 is embryonically lethal and the exact impact of cholesterol uptake via this receptor thus remains elusive. However, in our in vivo study the expression of LRP1 was equal between wild type and ApoE knockout mice (lacking ApoE as important ligand for LRP1-mediated uptake). The third route for adrenal cholesterol uptake is facilitated by SR-BI [41-44]. SR-BI is best known for its affinity for HDL and subsequent selective cholesteryl ester uptake in both the liver and adrenal. Humans with a functional mutation in the SR-BI gene (P297S missense mutation) display increased HDL-C levels and a decreased adrenal steroidogenesis [36]. Yet, the

contribution of SR-BI to cellular cholesterol uptake is not restricted to the HDL fraction but includes selective and whole particle uptake of VLDL/LDL for the acquisition of cholesteryl esters [45-48]. We therefore hypothesize that uptake of VLDL/LDL-derived cholesteryl esters by murine adrenals is also facilitated by SR-BI (**Figure 2 and Chapter 4, Figure 5**). However, further studies need to be executed to elucidate the exact mechanism and its role and relevance for the human situation.

Whereas different circulating lipoprotein fractions affect the steroidogenesis rate, the distribution and presence of intracellular cholesterol could also be of importance. Interestingly, hyperlipidemic ApoE deficient mice exhibit unexplained hypercorticosteronemia. The abundant presence of extracellular substrate for the conversion into steroid hormones could partly explain the hypercorticosteronemia. However, in vitro studies have indicated that locally produced ApoE may also impact directly on adrenal glucocorticoid output. Prack et al. [49] as well as Reyland et al. [50] suggested that intracellular cholesterol transported by ApoE is directly regulating the production of glucocorticoids through interference in the adrenocorticotropic hormone (ACTH)-induced cyclic adenosine monophosphate (cAMP) response [51]. In **Chapter 5** we evaluated the specific effect of adrenal ApoE deficiency on adrenal cholesterol metabolism and steroidogenesis by transplantation of adrenals from ApoE knockout mice into adrenalectomized wild-type animals. The local deletion of adrenal ApoE did not affect glucocorticoid levels in the circulation but did result in the accumulation of cellular cholesterol which was paralleled by endoplasmic reticulum (ER) stress. We therefore hypothesize a novel role for ApoE in the adrenal whereby it actively mobilizes and efflux excess cholesterol to prevent the development of ER stress (**Figure 2**). Yet, further research is required to uncover the exact mechanism that underlies the suggested role of adrenal ApoE.

FUTURE PERSPECTIVES

Development of atherosclerosis and the related cardiovascular events are worldwide the number one cause of death [1]. Scientific milestones like the discovery of the link between cholesterol levels and lesion progression by Nikolay Anitschkow [52] and Russel Ross's establishment of the importance of the inflammatory component in the progression of atherosclerosis [53], largely increased the understanding of the pathobiology underlying the progression of atherosclerotic cardiovascular disease. Targeting a main risk factor, LDL-C, by the introduction of statin-based treatments in

large cohort studies revealed the possibility to reduce overall cardiovascular events and halt further progression of lesion formation. However, actual changes in lesion composition and size are modest with the current interventions [54-56]. Despite the effective statin-induced lowering of LDL-C, in a large group of high-risk patients statin monotherapies inadequately lower serum cholesterol. Furthermore, statin-induced side effects are frequently one of the reasons for discontinuation of the treatment [57]. Therefore, alternative non-statin based therapies have been developed to employ a further reduction in the CVD risk.

The discovery of the enzyme proprotein convertase subtilisin/kerxintype-9 (PCSK9) and the following development of monoclonal IgG2 antibody-based inhibitors of PCSK9 (Alirocumab and Evolocumab) that halt LDL receptor degradation and lower LDL-C [58-60] provided an alternative cholesterol lowering strategy that is effective on top of statin-treatment. The monoclonal antibody approach to silence PCSK9 significantly lowers the LDL-C by ~50 to 60 % and the risk for a cardiovascular event by 9.8 % (Evolocumab) or 15 % (Alirocumab). However, this class of drugs have a downside. High costs and the subcutaneous injections every 2 or 4 weeks made these therapies only available for a selective group of patients. Therefore, alternative therapeutics are under development to inhibit PCSK9 [61], including small interfering RNA (siRNA) [62], vaccine/virus-like particles derived peptides [63-65] and small molecules [66]. These approaches open up a new field to achieve CVD risk reduction via extreme LDL-C lowering therapies and will contribute to the discussion whether a "lower the better" cholesterol strategy will be the future of CVD risk reduction.

Despite the LDL-C lowering associated risk reduction of a CVD event, a residual risk remains. Since lesion progression is an interplay between cholesterol and the inflammatory response of innate and adaptive immune cells, it is hypothesized that it might be beneficial to target the immune response on top op cholesterol lowering. However, anti-inflammatory therapies including corticosteroids and non-steroidogenic anti-inflammatory drugs (NSAID) increase CVD events [67]. The first successful trial targeting the innate immune system, in addition to the conventional therapeutics (LDL-C lowering), was the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) trial by Novartis using an monoclonal antibody-based IL1β inhibitor. This anti-inflammatory treatment dose-dependently lowered IL-6 levels and reduced the risk for primary end points (MI, Stroke, and CVD-related death) while the LDL-C levels remain unchanged [68]. Interestingly, IL1β antibody interventions in ApoE knockout mice with late stage atherosclerosis revealed no effect on lesion size, but

a change in plaque morphology. IL1β antibody-treated mice showed a reduction in smooth muscle cells and collagen and an increased macrophage content [69]. This phenotype in humans could have a Janus faced effect, where in absence of a large collagen-rich cap macrophages can more easily egress from the lesions. Yet, a low collagen content and increases the instability of the lesion leading to more rupture prone plaques. In 2018 Novartis discontinued the development of Canakinumab based on the modest reduction in primary end points and high price per quality-adjusted life-year (QALY) gained ($6.4 million per QALY) [70]. Other alternative trails that test the "inflammatory hypothesis" include the COLCOT trial, using a cheaper and approved anti-inflammatory drug, colchicine. The outcome of this study is positive, with a risk reduction for ischemic cardiovascular related events in patients with a recent myocardial infarction [71]. However, extensive side effects might limit its use in CVD treatment. Overall, anti-inflammatory therapeutics that test the "inflammation hypothesis" could be beneficial in addition to the conventional therapies, however safety and cost effectiveness is a general concern.

Lesion regression of rupture-prone vulnerable / existing lesions includes size reduction (regression) as well as a favorable changes in morphology and composition of the lesions (stabilization). It was a long standing hypothesis that lesion regression of (advanced) plaques was not achievable due to irreversible elements in the composition (necrotic core, fibrosis and calcification). Since the turn of the century, animal models revealed that regression of existing rupture-prone vulnerable lesions was feasible [9,72]. In line, we learned from in vivo models that lesion regression is not simply the reversal of mechanisms that drive progression. It requires distinct processes that initiate the removal of either unwanted cell types or components out of the plaque area, leading to a more favourable composition or shrinkage of the lesion. In addition, non-invasive trials visualizing the lesion progression with IVUS, CIMT ultrasound or angiography showed that upon lipid lowering, lesions in the coronary artery transform into more stable and collagen-enriched lesions [73]. The composition of lesions in the carotid artery could be a predictive biomarker for future events throughout the vascular system [74]. Albeit that stabilization of existing lesions is anticipated to lower the risk for a CVD event, the ideal situation is to deplete all existing lesions from the arterial tree. This is a challenge for future treatments (if feasible at all) and will come with a variety of hurdles to take, including; avoiding atherothrombosis, removal of the collagen layer and the recovery of a healthy endothelial layer.

LABORATORY ANIMAL RESEARCH

For the studies described in this thesis the availability of different knockout mouse models and techniques to modulate gene expression via transplantation techniques have been instrumental. An estimated 14 - 16 million animals, of which 85 - 90 % rodents, are annually used for biomedical research purposes in the U.S. alone [75]. In the Netherlands approximately 530.000 animals, of which 305.000 rodents, were used in 2017 [76]. Despite the elaborate mechanistic lessons learned from the use of in vivo models, the overall problem is that one cannot simply extrapolate in vivo data from animal models towards the human situation. Therefore, the predictive power of animal models for novel drug-based interventions is questioned [77,78]. In the discussion around the predictive power of laboratory animal models, the evolutionary biology and thus genetic (in)equality plays a pinnacle role. Animals that are closely related to humans, the primates, are little used for biomedical research due to ethical objections and high housing costs. The most common animal species used for biomedical research are rodents as they are easy to handle, low in costs and reproduce fast [79]. Although it is clear that understanding a biological mechanism in one species could help to understand the process in a second, the complexity of the biological pathways make that the power of predictions between species for biological function is hard. For CVD, Pasterkamp et al executed a systematic review with the aim to validate atherosclerosis-related genes in ApoE and LDLr knockout mice versus their human orthologues. Interestingly, they found no association with the murine adaptive and innate immunity related genes versus human plaque characteristics, coronary artery disease (CAD) or large artery ischemic stroke (LAS). However, the murine lipid metabolism related genes were significantly associated with human CAD, which is in line with the known role of cholesterol and the risk for CVD. An explanation for the observed discrepancies could be that the reviewed mice models involved primarily total-body knockout mice while mutations for the same genes in humans could have a more mild effect [80]. Nevertheless, it is the complexity of the biological pathways in animals that gives the added value in research fields that cover complex pathologies, like CVD.

Replacement of animals in (drug) research gained interest by the scientific community. During the last decade the use of animals for research is committed to the 3R strategy of Refinement, Replacement, and Reduction. With the rapid progress in the stem cell field it is now possible to skew pluripotent stem cells into almost any cell type. In combination with 3D culturing of different cell types that re-aggregate into organ-like structures, organoids, a potential drug testing and organ replacement platform is created [81]. Furthermore, with the development of the organ-on-a-chip, a 3D

microfluidic based dynamic cell-interaction device [82], several organs and tissue types are mimicked. This platform is designed to function as a 3D in vitro organ model to predict e.g. the pharmacokinetics of drugs. The potential of both platforms could open-up an era where there will be a reduction and replacement of laboratory animals currently used to study complex pathologies. In line, the former Dutch state secretary of economics, Martijn van Dam, set out the ambition in 2016 to make the Netherlands the first animal model free country by 2035 [83]. Recently the U.S. Environmental Protection Agency (EPA) also announced that it will no longer fund studies executed on animals by 2035 [84]. The Dutch ambition was shaped by the public debate around the reduction of laboratory animals and upcoming new techniques. The Dutch committee "Nationaal Comite Advies Dierproevenbeleid (NCAD)" that was assigned to advise the ministry on this topic suggested that a substantial reduction in the use of laboratory animals is an achievable goal [83]. Starting with phasing out the by the government imposed regulatory safety testing and release of biological safety products. However, despite the powerful alternative models described above, the committee agrees that reduction of laboratory animals in pre-clinical and fundamental research cannot be followed at the same pace. Therefore the Dutch scientific community is requested to think along how to achieve this goal in the coming 10 years. It will be interesting to follow the innovations that make this transition possible. Yet, this transition should be accomplished without losing our current expert position in the international research field and coping with the need to study complex biological systems in vivo.

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