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Glucocorticoids are active players and therapeutic targets in atherosclerotic cardiovascular disease

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ABSTRACT

Adrenal-derived glucocorticoids mediate the physiological response to stress. Chronic disturbances in glucocorticoid homeostasis, i.e. in Addison's and Cushing's disease patients, predispose to the development of atherosclerotic cardiovascular disease. Here we review preclinical and clinical findings regarding the relation between changes in plasma glucocorticoid levels and the atherosclerosis extent. It appears that, although the altered glucocorticoid function can in most cases be restored in the different patient groups, current therapies do not necessarily reverse the associated risk for atherosclerotic cardiovascular disease. In our opinion much attention should therefore be given to the development of a Cushing's disease mouse model that can (1) effectively replicate the effect of hypercortisolemia on atherosclerosis outcome observed in humans and (2) be used to investigate, in a preclinical setting, the relative impact on atherosclerosis susceptibility of already available (e.g. metyrapone) and potentially novel (i.e. SR-BI activity modulators) therapeutic agents that target the adrenal glucocorticoid output.

1. Glucocorticoids: stress hormones that modulate inflammation and metabolism

Stress is defined as the body's reaction to an external stressor, i.e. an event that is a real or perceived threat to the human well-being. The hypothalamus-pituitary-adrenal (HPA)-axis plays an essential role in the biological response to stress as it initiates a hormone-driven reaction within the body that involves a change in the activity of a variety of organ systems. Upon exposure to the physiological or psychological stressor the hypothalamus starts to secrete high levels of corticotropin-releasing hormone (CRH) (Antoni, 1986; Aguilera, 1994). CRH subsequently binds to its receptors in the pituitary resulting in the production and release of the polypeptide adrenocorticotropic hormone (ACTH) (Antoni, 1986; Aguilera, 1994). The adrenals respond to the relatively high levels of ACTH in the blood circulation by stimulating the generation of the so-called stress hormones, i.e. glucocorticoids such as cortisol, that initiate the actual physiological response to the stress.

Glucocorticoids belong to the family of steroid hormones that are produced within the mitochondria from the common precursor cholesterol. The conversion of cholesterol into cortisol encompasses several steps that are mediated by cytochrome P450 monooxygenases such as the rate-limiting enzyme cholesterol side-chain cleavage enzyme (CYP11A1) as well as 3-beta-hydroxysteroid dehydrogenase type 2 (HSD3B2; Fig. 1). The relative importance of each step for the production of cortisol is highlighted by the fact that human carriers of functional mutations in the different cytochrome P450 enzymes or HSD3B2 suffer from adrenal insufficiency (Wijaya et al., 2019; Perry et al., 2005; Guran et al., 2016; Speiser, 2001). Adrenals of adult rodents lack the cytochrome P450 enzyme steroid 17alpha-monooxygenase (CYP17A1), also referred to as 17alpha-hydroxylase, 17,20-lyase, and 17,20-desmolase, with eliminates the possibility to convert cholesterol into cortisol (Perkins and Payne, 1988; Le Goascogne et al., 1991; Keeney et al., 1995). As such, as can be appreciated from Fig. 1, the adrenals from mice and rats produce the glucocorticoid species corticosterone.

Cortisol and corticosterone execute their biological stress response through an interaction with the cognate glucocorticoid receptor, the first identified member of the nuclear receptor superfamily of transcription factors (Hollenberg et al., 1985). The glucocorticoid receptor is ubiquitously expressed throughout the body with relatively high expression levels being found in the brain as well as in a variety of immune cells/tissues (Bookout et al., 2006). Within the brain, the glucocorticoid receptor mediates the negative feedback control of glucocorticoids on the HPA-axis activity (Reul and de Kloet, 1986; Diorio et al., 1993; Radley et al., 2006). Activation of the glucocorticoid receptor in immune cells is generally associated with immune suppression

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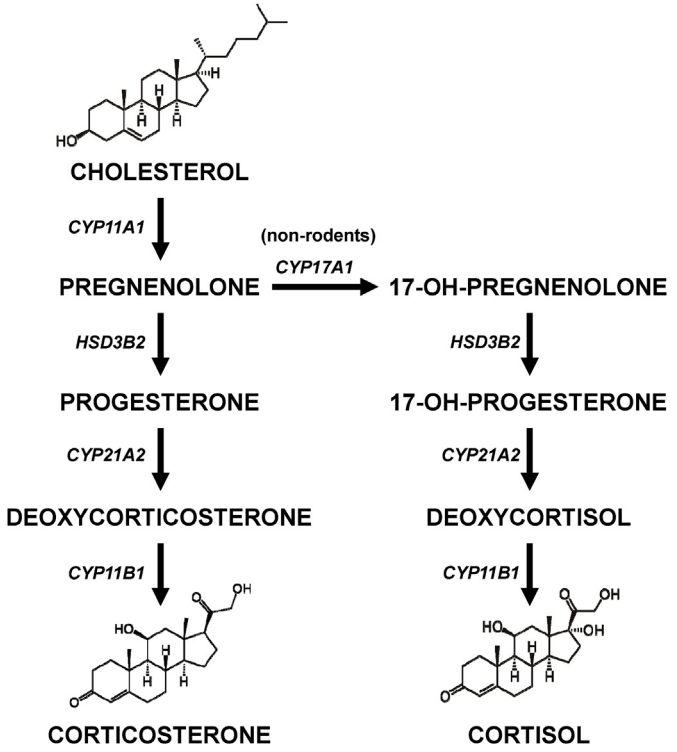

Fig. 1. Schematic representation of the steps involved in the conversion of cholesterol into the main circulating glucocorticoid species cortisol (non-rodents) and corticosterone (rodents). CYP11A1, side-chain cleavage enzyme. HSD3B2, 3-beta-hydroxysteroid dehydrogenase type 2. CYP21A2, 21-hydroxylase. CYP11B1, 11-beta-hydroxylase. CYP17A1, steroid 17alpha-monooxygenase.

as a result of the transrepressive action on its target genes. Functional antagonism exists between the glucocorticoid receptor and c-Jun, resulting in a decreased activator protein 1 (AP1)-mediated transcriptional activation in immune cells in response to treatment with a glucocorticoid receptor ligand (Schüle et al., 1990). As a result, glucocorticoids inhibit pro-inflammatory interferon signaling in peripheral blood monocytes through a glucocorticoid receptor-mediated decrease in signal transducer and activator of transcription 1 (STAT1) production (Hu et al., 2003). Glucocorticoids are also able to suppress the secretion of the pro-inflammatory cytokines tumor necrosis factor-alpha and interleukin-12 by monocytes/macrophages (Blotta et al., 1997; Steer et al., 2000; Ma et al., 2004). Furthermore, the viability and activation state of T cells is reduced through exposure to glucocorticoids translating into a reduced production and action of the pro-inflammatory cytokine interleukin-2 (Vacca et al., 1990; Kiefer et al., 1995; Helmberg et al., 1995). Despite the fact that mRNA expression levels of the glucocorticoid receptor are generally lower in metabolic tissues than those found in tissues of the immune system, i.e. thymus and spleen (Bookout et al., 2006), the relatively high levels of glucocorticoids found under stress conditions can also significantly influence metabolism. Expression of the glucocorticoid receptor within the liver is essential for survival as judged from the observation that mice lacking functional expression of the glucocorticoid receptor specifically only in hepatocytes exhibit severe growth deficits and die shortly after birth (Tronche et al., 2004). The hepatic glucocorticoid receptor controls transcription of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK; Imai et al., 1990), which contributes to the maintenance of blood glucose levels under fasting stress conditions (Opherk et al., 2004). Their action in adipose tissue also contributes to the effect of glucocorticoids on blood glucose levels. More specifically, glucocorticoid exposure increases the expression of the glucocorticoid receptor target gene mitogen-activated protein kinase 1, which is paralleled by diminished insulin-stimulated glucose uptake by adipocytes (Shipp et al., 2010; Tchen et al., 2010). Glucocorticoids also play a significant role in the control of adipocyte triglyceride metabolism as they, through the action of the glucocorticoid receptor, control the expression of the key lipolysis genes hormone-sensitive lipase (HSL), adipocyte triglyceride lipase (ATGL) and monoglyceride lipase (MGL; Villena et al., 2004; Yu et al., 2010).

2. Changes in the glucocorticoid status are associated with an increased risk for the development of atherosclerotic cardiovascular disease in humans

Given that glucocorticoids influence many different physiological processes it is not surprising that chronic changes in plasma glucocorticoid levels underlie the development of certain pathologies. As mentioned before, a genetic defect in the enzymes involved in the conversion of cholesterol into cortisol is associated with adrenal insufficiency. It should be acknowledged that the frequency of mutations in for instance 21-hydroxylase (21-OH; CYP21A2) is relatively low, with an incidence of 1/10000–1/20000 newborns (El-Maouche et al., 2017). As such, adrenal insufficiency is considered to be a rare disease. Subjects carrying a mutation in the CYP21A2 gene generally display glucocorticoid and/or mineralocorticoid (i.e. aldosterone) deficiency (El-Maouche et al., 2017). This pathology is also known as congenital adrenal hyperplasia (CAH) since the CYP21A2 deficiency is paralleled by adrenal hyperplasia due to chronically high plasma ACTH levels as a result of the diminished glucocorticoid-mediated negative feedback on the HPA-axis. The overall clinical phenotype can vary significantly between individual CAH patients with respect to the disease severity, since the extent of enzyme function impairment is dependent on the actual change in the CYP21A2 protein structure/availability. The classical form of CYP21A2 deficiency, i.e. characterized by both glucocorticoid and aldosterone insufficiency, commonly presents in infancy as a result of salt-wasting and the development of a life-threatening adrenal crisis rapidly after birth (Speiser et al., 2010). In contrast, mutations that result in a relatively mild impairment of CYP21A2 functionality are usually diagnosed during puberty or in (early) adulthood due to ACTH-induced overproduction of androgens by the adrenals. Human subjects suffering from non-classical CYP21A2 deficiency can display precocious pubarche, hirsutism, oligomenorrhea/amenorrhea, and female infertility (El-Maouche et al., 2017). Given that many of the developmental phenotypes found in CAH patients are secondary to the reduced ability of the adrenals to secrete glucocorticoids and mineralocorticoids, hormone replacement therapy serves as the first line treatment of subjects suffering from CAH.

Evidence is accumulating that adults suffering from CAH may also be at increased risk of developing the metabolic syndrome, a pathology characterized by at least three of the five following medical conditions: central obesity, high blood pressure, high blood glucose levels, high plasma triglycerides, and low plasma high-density lipoprotein (HDL)-cholesterol levels. A recent review of literature regarding the relation between CAH and changes in metabolic status by Improda et al. (2017) has suggested that children and adolescents with CYP21A2 deficiency-driven CAH are more prone to suffer from hypertension and obesity and display a higher susceptibility for the development of insulin resistance and possibly type II diabetes (independent of the obesity phenotype). However, Improda et al. (2017) have concluded based upon the reviewed studies that the presence of CAH does not impact significantly on the plasma lipid profile. All 5 abovementioned conditions associated with the metabolic syndrome individually increase the risk for the development of cardiovascular disease, with the combination translating into a ~2-fold higher chance of suffering from a myocardial infarction or stroke (Ninomiya et al., 2004).

Atherosclerosis, narrowing of the vessel lumen due to accumulation of cholesterol and other lipoid substances in so-called macrophage foam cells within the vessel wall, represents the primary underlying cause of

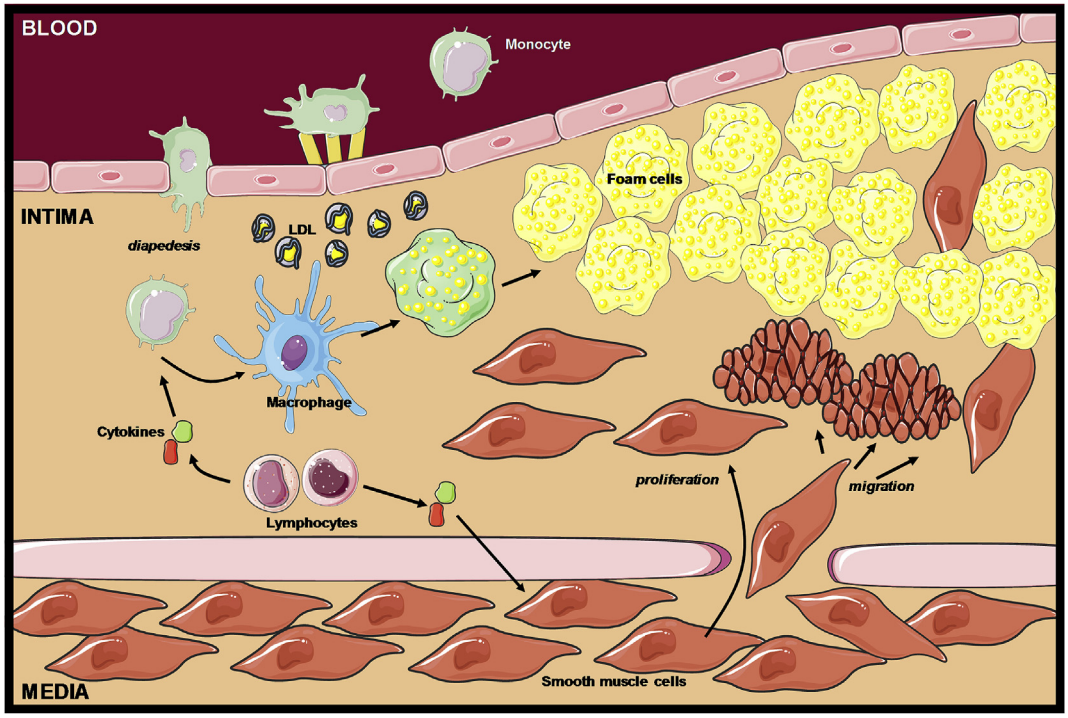

Fig. 2. Overview of key processes involved in the initial development and progression of atherosclerotic lesions. A combination of relatively high plasma LDL-cholesterol concentrations and other cardiovascular disease risk factors such as smoking induce damage to the endothelium which results in monocyte infiltration into the vessel well. Monocytes are locally converted into macrophages that can engulf LDL particles to become foam cells which leads to fatty streak lesion formation. In response to the chronic inflammation, e.g. cytokine secretion by attracted lymphocytes, smooth muscle cells migrate, proliferate and change phenotype to produce collagen, which enables the formation of a fibrous cap covering the more advanced plaque.

cardiovascular disease (Ross, 1986; Ross and Glomset, 1976a and 1976b). As such, lipid-lowering statin treatment constitutes the first line therapy for subjects considered at high risk of developing cardiovascular disease. Although arterial lipid deposition in macrophage foam cells is a hallmark of the development of atherosclerotic lesions, it should be acknowledged that the pathogenesis of atherosclerosis is also characterized by endothelial cell dysfunction, leukocyte infiltration, cytokine-driven inflammation, and smooth muscle cell migration and proliferation (Fig. 2) (Ross, 1986; Ross and Glomset, 1976a/b). The relative importance of the inflammatory component in atherosclerotic cardiovascular disease has become apparent in the recent CANTOS trial which showed a beneficial impact of pro-inflammatory cytokine interleukin-1beta (IL-1beta) inhibition on cardiovascular disease outcome that was independent of cholesterol lowering (Ridker et al., 2017).

In accordance with the general assumption that presence of the metabolic syndrome increases the risk of atherosclerotic cardiovascular disease, the relatively high metabolic syndrome predisposition of CAH patients is associated with a concomitantly higher susceptibility for the development of atherosclerosis. More specifically, Amr et al. (2014), Akyürek et al. (2015), and Metwalley et al. (2016) observed that children suffering from CAH exhibited a higher atherosclerosis extent as compared to age-matched unaffected human control subjects, as judged by their higher carotid intima-media thickness. In support, Sartorato et al. (2007) have also detected a relatively higher intima-media thickness in CAH patients compared to healthy controls in adulthood. A meta-analysis of 12 longitudinal and two cross-sectional studies that included data from 437 CAH patients, covering both children and adult populations, revealed that the intima thickness is on average 0.08 mm larger in patients than in healthy individuals without CAH (Tamhane et al., 2018).

The higher cardiovascular disease susceptibility does not appear to be restricted to CAH patients, but rather appears a common finding in subjects suffering from (primary) adrenal insufficiency. A study by Bergthorsdottir et al. (2006) indicated that the relative risk score for all-cause mortality is > 2-fold higher in Addison's disease patients with primary adrenal insufficiency, which can be primarily related to a significant increase in cardiovascular disease susceptibility. Skov et al. (2019) also observed that cardiovascular disease risk was higher in Addison's disease patients as compared to healthy controls. An interesting point that can be derived from the data of Skov et al. (2019) is that the cardiovascular predisposition associated with Addison's disease may be much higher in female than in male patients. However, the underlying reason for this apparent gender bias remains to be uncovered. Notably, the negative effect on overall cardiovascular disease risk appears to be driven by an effect on ischemic heart disease and cannot be attributed to a rise in the risk for the development of cerebrovascular disease (Skov et al., 2019).

Improda et al. (2017) have implied that high extent hormone replacement contributes significantly to the metabolic disturbances, increase in blood pressure and the associated cardiovascular disease risk found in CAH patients. In agreement, a positive relationship was detected between the hydrocortisone and fludrocortisone dose used and cardiovascular disease risk in the cohort studied by Skov et al. (2019), with intake of the highest doses of both hydrocortisone and fludrocortisone translating into a ≥2-fold increase in the odds ratio for cardiovascular disease in both men and women. It should be noted that the hormone treatment-associated change in the metabolic status alone does, however, not fully explain the overall increased mortality rate of human subjects suffering from CAH and Addison's disease, since Chantzichristos et al. (2017) observed that primary adrenal insufficiency even further reduces the life expectancy also in an all diabetic population.

The notion that relatively high plasma glucocorticoid levels are a direct cause of metabolic complications and cardiovascular disease is supported by findings from human subjects suffering from Cushing's syndrome. Cushing's syndrome is a rare, chronic disease characterized

by endogenous or exogenous hypercortisolemia. Cushing's disease, i.e. the presence of an ACTH-secreting tumor in the pituitary which overrules the glucocorticoid-mediated feedback on the HPA-axis, represents the primary underlying cause of the hypercortisolemia (70% of all Cushing's cases; Nieman et al., 2015; Pivonello et al., 2017). Early signs of the presence of Cushing's syndrome include easy bruising, facial plethora, proximal myopathy (or proximal muscle weakness), and the development of striae (Nieman et al., 2008). In children, Cushing's syndrome can also be apparent from excessive weight gain (obesity) in the context of decreasing growth velocity (Nieman et al., 2008). However, the prevalence of hypertension, glucose intolerance, and diabetes is also generally higher in Cushing's syndrome patients than in non-diseased human subjects (Nieman et al., 2008; Feelders et al., 2012; Pivonello et al., 2017). Plotz et al. already in 1952 observed that the highest mortality amongst Cushing's syndrome patients is due to either infections or cardiovascular diseases, i.e. cardiac failure. In accordance, a 5 times higher vascular disease mortality rate was detected by Etxabe and Vazquez (1994) in subjects affected by Cushing's disease. About similar increases in the relative risk for the development of a myocardial infarction or stroke were described by Lindholm et al. (2001) and Dekkers et al. (2013) after intensively following the clinical course of two independent cohorts of Cushing's syndrome patients. Clayton et al. (2011) even found a 13 times higher mortality rate related to vascular complications in Cushing's patients. A meta-analysis by Clayton et al. (2011) on the outcomes of 4 different studies investigating the impact of Cushing's syndrome on overall mortality has indicated that cardiovascular disease-related mortality rates are ~5.5-fold higher in Cushing's syndrome patients than in the general population. Studies by Dalmazi et al. in a cohort of human subjects with incidental adrenal tumors have suggested that even a minor, chronic increase in plasma cortisol levels translated into an increased risk of cardiovascular events and mortality (Di Dalmazi et al., 2014). The increase in overall cardiovascular disease (mortality) risk in Cushing's syndrome patients is probably secondary to an increased atherosclerotic plaque burden, given that a meta-analysis by Lupoli et al. (2017) has indicated that both the carotid intima-media thickness and the incidence of carotid plaques are significantly higher in subjects suffering from Cushing's than in healthy controls. In line with the overall conclusion that chronic glucocorticoid overexposure predisposes humans to atherosclerotic cardiovascular disease, stress has also been shown to aggravate cardiovascular disease burden. A 27 year follow-up of 136.673 Swedish individuals showed that the cardiovascular disease incidence was 1.64 higher in subjects that were diagnosed for a stress-related condition as compared to non-stressed control subjects (Song et al., 2019). In addition, patients with stable coronary artery disease that persistently experience moderate or greater psychological stress display an almost 4-fold increase in cardiovascular mortality risk (Stewart et al., 2017).

3. Glucocorticoids elicit variable effects on atherosclerosis burden in preclinical animal models

The first animal studies regarding the ability of glucocorticoids to modulate atherosclerosis susceptibility were executed in rabbits in the 1960s, because rabbits rapidly develop atherosclerotic lesions within the thoracic aorta in response to feeding them a diet enriched in cholesterol. In contrast to what would be expected from the atherosclerosis stimulating effect of Cushing's syndrome in humans, Bailey and Butler showed that high dose oral administration of a variety of glucocorticoid species, i.e. dexamethasone, 9-alpha-fluorohydrocortisone/fludrocortisone, and methylprednisolone, significantly reduces plaque load in high cholesterol diet-fed New Zealand White rabbits (Bailey and Butler, 1973). The positive effect of glucocorticoid treatment on atherosclerosis outcome was suggested to be primarily related to the anti-inflammatory actions of the respective glucocorticoid species, since non-steroidal anti-inflammatory drugs were also capable of reducing atherosclerosis susceptibility in a similar experimental setting (Bailey and Butler, 1973). Asai et al. (1993) and Naito et al. (1992) observed that dexamethasone was also able to execute its atherosclerosis-reducing effect in New Zealand White rabbits when administered through intra-muscular injection. The decrease in atherosclerosis burden in dexamethasone-treated rabbits in the study by Asai et al. (1993) coincided with a reduction in the lesional macrophage and lymphocyte content. Notably, findings from Hagihara et al. (1991) have shown that dexamethasone treatment markedly lowers the adhesion of leukocytes to the aortic endothelium, which also explains the dexamethasone-induced protection to cuff-induced intimal thickening observed in Japanese white rabbits. Anti-inflammatory glucocorticoid administration to rabbits, i.e. oral prednisone treatment, similarly reduces the restenosis extent in response to stent implantation into atherosclerosis-containing iliac arteries (Ribichini et al., 2007). In accordance with the notion that potent anti-inflammatory actions drive the glucocorticoid-mediated atheroprotection, the positive effect on balloon injury-induced restenosis upon dexamethasone treatment appears to be related to a reduced monocyte chemoattractant protein-1 (MCP-1)-mediated attraction of lymphocytes towards the injured vessel wall (Poon et al., 2001). Data from Cavallero et al. (1976) have highlighted that the glucocorticoid-induced reduction in atherosclerosis susceptibility in rabbits is possibly due to local actions on cells within the atherosclerotic plaque, i.e. through a reduction in the proliferation rate of smooth muscle cells and/or infiltrated immune cells. In support, liposome-mediated local delivery of a single glucocorticoid dose to macrophages into atherosclerotic plaques results in a diminished lesion inflammatory status, as judged by the observed reduction in aortic ^{18}F-fluoro-deoxy-glucose uptake in prednisolone phosphate liposome-administered New Zealand White rabbits (Lobatto et al., 2010). Hypercholesterolemia is a determinant factor in the development of atherosclerotic lesions in New Zealand White rabbits based upon the observation that plasma cholesterol levels correlate significantly to total lesion area and the lesional cholesterol contents (Shaish et al., 1997). Findings from Tvedegaard et al. (1983) have suggested that, apart from the anti-atherogenic anti-inflammatory effect, glucocorticoid treatment inhibits the formation of atherosclerotic lesions through reducing the aortic uptake of cholesterol from the plasma compartment. As such, no increased atherosclerosis burden was measured in their methylprednisone-treated rabbits despite the significant increase in the plasma hyperlipidemia extent after high dose methylprednisone treatment (Tvedegaard et al., 1983).

Wild-type mice are resistant to the development of atherosclerotic lesions, as their plasma levels of pro-atherogenic, cholesterol-rich low-density lipoproteins (LDL) remain too low to initiate the atherogenesis process even after high cholesterol diet feeding (Ishibashi et al., 1994). However, in the earlier 90's, two different lines of genetically modified mice were generated that exhibit a high susceptibility for the development of atherosclerotic lesions. The group of Dr. Nobuyo Maeda have developed a strain of apolipoprotein E (APOE) knockout mice that display spontaneous arterial lesions, since the genetic lack of APOE protein is associated with the accumulation of cholesterol-rich very-low-density lipoprotein (VLDL) and chylomicron remnants in the blood compartment (Zhang et al., 1992). In contrast, mice with a genetic deficiency of the LDL receptor (LDLR), generated by Dr. Joachim Herz and colleagues (Ishibashi et al., 1993), only display mild hypercholesterolemia upon regular chow diet feeding with a comparable lipoprotein profile as normally found in humans. LDLR knockout mice therefore require dietary cholesterol supplementation to facilitate the development of atherosclerotic lesions (Ishibashi et al., 1993). With the introduction of these new animal models for human cardiovascular disease, a concomitant shift from rabbits to mice occurred in the choice of the species used for (in vivo) studies regarding the role of glucocorticoids in the atherosclerosis pathology. Stein et al. (1998) showed that glucocorticoid treatment of mice was associated with a diminished flux of cholesterol back to the liver for subsequent excretion into the bile (reverse cholesterol transport), which translated into a prolonged

accumulation of LDL-cholesterol in the injection compartment. This initial finding in mice suggested that mice as compared to rabbits would probably serve as better model to investigate the effect of glucocorticoids on human atherosclerosis disease. Within cells targeted by glucocorticoids, i.e. hepatocytes and macrophages, the enzyme 11beta-hydroxysteroid dehydrogenase type 1 (HSD11B1) can convert inactive cortisone into active cortisol, thereby raising the effective cellular glucocorticoid tone. This action is counterbalanced by the enzyme HSD11B2 that inactivates cortisol through converting it back to cortisone. Hermanowski-Vosatka et al. (2005) observed that pharmacological inhibition of the HSD11B1 activity was associated with a diminished atherosclerotic lesion growth in APOE knockout mice, fueling the suggestion that glucocorticoids should be considered pro-atherogenic. Treatment with HSD11B1 inhibitors or a genetic deletion of HSD11B1 protein function similarly reduced the extent or aortic cholesterol accumulation in Western-type diet-fed APOE knockout mice (Luo et al., 2013; García et al., 2013) and severely obese A^{y}/a LDLR knockout mice (Nuotio-Antar et al., 2007). It has been suggested that the atherosclerosis reducing effect of HSD11B1 deficiency can for the large part be attributed to a beneficial impact on lesional macrophage cholesterol handling (Kipari et al., 2013; García et al., 2013). In this context it is interesting to note that van der Valk et al. (2016) have shown that liposome-mediated local delivery of prednisolone to plaque macrophages is associated with accelerated atherosclerosis in LDLR knockout mice, as expected. In accordance with a pro-atherogenic role for glucocorticoids, Schepers et al. (2006) detected a > 2-fold rise in plasma cholesterol levels and a concomitant 4.7-fold increase in the atherosclerotic plaque area in the aortic root of dexamethasone-treated genetically modified hyperlipidemic APOE3Leiden mice. In striking contrast to the notion that (excess) glucocorticoids execute a pro-atherogenic effect in mice, high dose dexamethasone treatment reduced the arterial cholesterol load in wild-type mice fed a cholesterol and cholic acid-enriched atherogenic diet despite the fact that glucocorticoid exposure was associated with exacerbated hypercholesterolemia (Tauchi et al., 2001). Studies by Auvinen et al. (2013) in APOE3Leiden mice also carrying the human cholesteryl ester transfer protein (CETP) transgene have shown a similar atheroprotective effect of both transient and continuous glucocorticoid excess. Furthermore, APOE knockout mice lacking the glucocorticoid receptor in their endothelial cells developed more severe atherosclerotic lesions in the aorta, the brachiocephalic artery, as well as in the aortic sinus (Goodwin et al., 2015). In support of a highly variable effect of glucocorticoids on atherosclerosis outcome, HSD11B1 inhibitor treatment did not modulate atherosclerosis susceptibility in Western-type diet fed LDLR knockout mice (Nuotio-Antar et al., 2007). In addition, HSD11B1 inhibition did not reduce the aortic plaque load in chow diet-fed leptin deficient APOB100 transgenic obese LDLR knockout mice (Lloyd et al., 2009). A selective disruption of glucocorticoid receptor functioning in macrophages in LDLR knockout mice (Preusch et al., 2008) or treatment of high fat diet-fed APOE knockout mice with dexamethasone (Zhang et al., 2015) did also not translate into an altered atherosclerosis susceptibility. As recently observed by our group, the cholestasis-associated hypercorticosteronemia also fails to impact atherosclerosis extent in APOE knockout mice (van der Geest et al., 2019). Furthermore, we have previously noted that a complete loss of adrenal glucocorticoid production as a result of bilateral adrenalectomy is not associated with an effect on atherosclerosis susceptibility in APOE knockout mice (Hoekstra et al., 2013c), while it actually increases the susceptibility to the development of atherosclerotic lesions in LDLR knockout mice fed a pro-inflammatory cholic acid-containing Western-type diet (van der Sluis et al., 2012). These combined findings indicate that (1) the effect of (endogenous) glucocorticoids on disease outcome in atherosclerosis mouse models is very variable and (2) it is worthwhile to put effort in the generation of predictive Cushing's syndrome-related atherosclerosis mouse models.

Zebrafish have emerged as a low-cost, non-mammalian preclinical model system that can perhaps also be used to study the interaction between glucocorticoids and atherosclerotic cardiovascular disease. Glucocorticoid functionality in zebrafish mimics that of humans at several important levels, as reviewed by Schaaf et al. (2009). For instance, as opposed to mice, adrenals from zebrafish express a functional CYP17 protein and are therefore able to produce cortisol (Wang and Ge, 2004). Furthermore, zebrafish ubiquitously express both the alpha and beta isoforms of the glucocorticoid receptor, with the alpha subtype exhibiting the highest relative tissue expression levels (Schaaf et al., 2008). An interesting aspect from applying zebrafish as potential atherosclerosis animal model is that disease progression can theoretically be followed in living animals, i.e. through in vivo imaging of fluorescently-labeled macrophages, given that zebrafish bodies are optically transparent. Feeding a diet enriched in cholesterol effectively induces hypercholesterolemia in zebrafish (Schlegel, 2016). The Miller group was the first to suggest that this hypercholesterolemia also translates into the deposition of lipids within the arterial vessel wall (Stoletov et al., 2009). However, in spite of multiple attempts, we have not been able to verify that the cholesterol enriched diet-induced hypercholesterolemia is indeed able to stimulate the formation of foam cell-rich lesions in zebrafish (unpublished data Verwilligen et al.). We therefore consider the qualification of zebrafish as established atherosclerosis animal model controversial. In this context, it is also important to acknowledge that recent experimental evidence from Luo et al. (2019) suggests that other myeloid cells (i.e. neutrophils) than macrophages - typically present in human atherosclerotic lesions - accumulate in the vessel wall of hypercholesterolemic zebrafish. More detailed research into this specific topic is clearly warranted.

4. Impact of current treatments for hypercortisolemia on (atherosclerotic) cardiovascular disease risk

Although the underlying disease mechanism can be quite different between individual Cushing's syndrome patients that suffer from hypercortisolemia, most disease cases can be treated or cured (Nieman et al., 2015). Treatment regimens with exogenous glucocorticoids as part of for instance anti-inflammatory rheumatoid arthritis therapies can be stopped or adapted to reverse the Cushing's syndrome-associated symptoms, whilst ACTH-producing pituitary tumors are treated through a combination of surgery, chemotherapy, and/or radiation (Nieman et al., 2015). Furthermore, Cushing's syndrome patients are prescribed steroidogenesis blockers such as the 11-beta-hydroxylase (CYP11B1) inhibitor metyrapone or glucocorticoid receptor antagonists, i.e. RU-486 (mifepristone) to respectively reduce the adrenal glucocorticoid output or diminish the metabolic effects associated with relatively high plasma cortisol levels (nicely reviewed by Cuevas-Ramos and Fleseriu, 2014). A retrospective multi-center study published in 2015 has shown that metyrapone treatment is an effective therapy for short- and long-term control of hypercortisolemia in Cushing's syndrome with > 50% of the patients reaching the (normalized) cortisol target of less than 300 nM/10.9 μg/dl (Daniel et al., 2015). However, it should be acknowledged that several side effects related to metyrapone treatment (acne, hirsutism, lethargy, dizziness, ataxia, nausea, hypertensive crisis, hypokalemia, edema, and adrenal insufficiency) contribute to a limited availability or use of metyrapone in most countries (Cuevas-Ramos and Fleseriu, 2014). Interestingly, despite effective treatment of the hypercortisolemia, the increased cardiovascular risk is not reversed in Cushing's syndrome patients after 1 year or 5 years of remission (Faggiano et al., 2003; Colao et al., 1999; Webb and Valassi, 2018). This appears to be related to the fact that metabolic complications associated with active Cushing's syndrome such as relatively high blood pressure, altered plasma lipid levels and obesity are not fully resolved through current cortisol lowering treatments (Faggiano et al., 2003; Colao et al., 1999; Barahona et al., 2009; Webb and Valassi, 2018). Glucocorticoid secretion by the adrenals normally follows an ultradian rhythm (Stavreva et al., 2009). Importantly, preclinical studies by

Sarabdjitsingh et al. (2010) have observed a significant flattening of the plasma glucocorticoid rhythmicity upon chronic glucocorticoid receptor overactivation in rats. It is therefore highly likely that ineffective restoration of plasma glucocorticoid rhythmicity also partially underlies the inability of the indicated treatments to completely reverse all complications related to the hypercortisolemia. Pro-thrombotic and pro-inflammatory factors that can influence the overall atherosclerotic cardiovascular disease risk also remain significantly higher in the circulation during Cushing's syndrome remission (Aranda et al., 2018; Barahona et al., 2009; Webb and Valassi, 2018). From large-scale clinical trials is has become evident that both lipid lowering treatments, i.e. statins, as well as anti-inflammatory approaches can serve as therapy for subjects at risk of developing atherosclerotic cardiovascular disease (Boekholdt et al., 2014; Ridker et al., 2017). It will be interesting to validate whether any of these approaches is able to eliminate the residual cardiovascular risk in Cushing's syndrome patients in remission. However, in light of the limited number of new Cushing's syndrome cases identified annually (incidence: 3.2 cases per one million inhabitants (Wengander et al., 2019), it may be difficult or even impossible to compile a cohort of patients that is sufficiently large to provide an answer to this question.

5. Adrenal cholesterol acquisition by scavenger receptor BI (SR-BI): a novel therapeutic target in treatment of Cushing's syndrome?

Cortisol synthesis intermediates such as 11-dexoycortisol can be converted into androgen precursors, leading to off-target effects related to androgen excess upon chronic treatment of Cushing's syndrome patients with standard steroidogenesis inhibiting drugs, i.e. metyrapone (Cuevas-Ramos and Fleseriu, 2014). Modulation of the adrenal cholesterol substrate availability could theoretically serve as an alternative to inhibiting individual steps in the conversion of cholesterol into cortisol to overcome hypercortisolemia. It has become evident from studies in both mice and humans that the adrenal cholesterol pool used for the synthesis of glucocorticoids is derived from either the novo cholesterol synthesis within the adrenals or via SR-BI- or LDLR-mediated uptake of cholesterol from circulating lipoproteins, with the latter being the primary acquisition route (Fig. 3). Studies in rats have shown that the basal activity of the rate-limiting enzyme in de novo cholesterol synthesis, HMG-CoA reductase (HMGCR), within the adrenals is low (Balasubramaniam et al., 1977). In further support, little to no adrenal activity of HMGCR has been found in normolipidemic wild-type mice (Plump et al., 1996). As such, pharmacological inhibition of the HMGCR activity through statin treatment does not impact plasma cortisol levels in (hypercholesterolemic) humans (Illingworth and Corbin, 1985; Laue et al., 1987; Fojo et al., 1987; Mol et al., 1989; Ide et al., 1990; Takeda et al., 1991; Azzarito et al., 1992). HMGCR expression and activity levels are markedly increased in the adrenals of mice in response to the absence of cholesterol-containing lipoproteins (Plump et al., 1996; Hoekstra et al., 2010), suggesting that de novo synthesis can make a significant contribution to adrenal steroidogenesis. In agreement, simvastatin treatment further reduces the already lowered adrenal glucocorticoid output in hypocholesterolemic APOA1 knockout mice (Ouweneel et al., 2017). Based upon the combined observations that cholesterol ester stores within the adrenals from HDL deficient APOA1 knockout mice are almost fully deprived and that these mice suffer from relative glucocorticoid insufficiency (Plump et al., 1996), it was long hypothesized that HDL should be regard as the primary source of the steroidogenic cholesterol pool. This suggestion was supported by the finding that adrenal cholesterol stores and the overall adrenal glucocorticoid function are similarly reduced in lecithin–cholesterol acyltransferase (LCAT) knockout mice, which are unable to convert pre-beta HDL particles into cholesterol ester-enriched mature HDL particles, as well as in human subjects carrying a functional mutation in the LCAT gene (Hoekstra et al., 2013b; Bochem et al., 2013). In contrast, follow-up studies in chow diet-fed LDLR knockout mice that exhibit a human-like lipoprotein profile have shown that HDL is actually redundant for adrenal steroidogenesis (Hoekstra and Van Eck, 2016). It can thus be anticipated that, in humans, non-HDL lipoproteins such as the APOB-containing lipoproteins VLDL and LDL are of quantitative importance as adrenal cholesterol donors. This hypothesis fits with clinical data from Illingworth et al. that ACTH-stimulated cortisol secretion is lower in abetalipoproteinemic (LDL deficient) patients as compared to unaffected controls (Illingworth et al., 1980, 1982a; 1982b). Given that hypercholesterolemic APOE knockout mice display hypercorticosteronemia (Raber et al., 2000), an effect that is not driven by elimination of adrenocortical APOE production (van der Sluis et al., 2019), mechanistic studies in total body APOE knockout mice can possibly shed more light on the importance of APOB-containing lipoproteins in the context of adrenal steroidogenesis.

SR-BI is predominantly known for its role in HDL metabolism. The selective uptake of cholesterol esters from HDL particles by the liver and adrenals is impaired in SR-BI knockout mice (Out et al., 2004a; Brodeur et al., 2005). As a result of the accumulation of cholesterol ester-enriched large HDL particles in the circulation, plasma HDL-cholesterol levels are significantly higher in SR-BI knockout mice as compared to wild-type littermates (Rigotti et al., 1997). Human carriers of a functional mutation in the SR-BI gene are also characterized by elevated plasma HDL-cholesterol levels (Vergeer et al., 2011; Zanoni et al., 2016; Helgadottir et al., 2018). However, SR-BI is also able to facilitate the cellular delivery of cholesterol from non-HDL APOB-containing lipoprotein species such a chylomicrons (Out et al., 2004b), VLDL (Van Eck et al., 2008) and LDL (Swarnakar et al., 1999; Stangl et al., 1999; Brodeur et al., 2005). In this context, it is of interest to note that a large body of evidence has indicated that - next to hypocholesterolemia - a disruption in the functionality of the scavenger receptor BI can also underlie an impaired glucocorticoid response in mice and humans. More specifically, total body SR-BI knockout mice fail to increase their plasma corticosterone concentrations in response to a LPS stress trigger to such a level that they can overcome the development of septic shock (Cai et al., 2008; Hoekstra et al., 2009). As a result, a markedly higher lethality rate is observed in SR-BI knockout mice than in control mice upon high dose LPS exposure (Cai et al., 2008). A significantly diminished rise in plasma corticosterone levels is also detected in total body and adrenal-specific SR-BI knockout mice as compared to their wild-type controls upon exposing them to fasting stress (overnight food deprivation) (Hoekstra et al., 2008, 2009, 2013a). The adrenal steroidogenesis capacity is also significantly attenuated in human subjects carrying the P297S functional mutation in the SR-BI gene, as judged by the finding that the urinary excretion of sterol metabolites and cortisol response to ACTH stimulation is reduced in P297S mutation carriers as compared to unaffected human controls (Vergeer et al., 2011). Notably, human SRBI P297S mutation carriers frequently show clinical signs related to adrenal insufficiency (Vergeer et al., 2011). Given that baseline plasma glucocorticoid levels are not reduced as a result of SR-BI deficiency (Hoekstra et al., 2008, 2009; 2013a; Vergeer et al., 2011), it can be anticipated that adrenocortical cell SR-BI plays an essential role specifically in the ACTH-mediated glucocorticoid response in mice and humans. Since the majority of clinical Cushing's cases are resulting from ACTH-mediated adrenal overstimulation, i.e. ACTH-producing pituitary tumors, it can be suggested that targeting SR-BI functionality may be of use in the treatment of Cushing's syndrome patients. Importantly, SR-BI contributes to the cellular entry of hepatitis C virus (Westhaus et al., 2017; Gao et al., 2017) and SR-BI blocking agents, i.e. the antagonist ITX5061, are therefore currently being developed as anti-hepatitis C drugs (Syder et al., 2011; Rowe et al., 2016). Since ITX5061 has been shown to be well tolerated with measurable plasma concentrations during short-term therapy in patients with hepatitis C virus infection undergoing liver transplantation (Rowe et al., 2016), we consider it clearly of interest to evaluate the effect of ITX5061 treatment on the adrenal steroid function in both healthy human subjects as

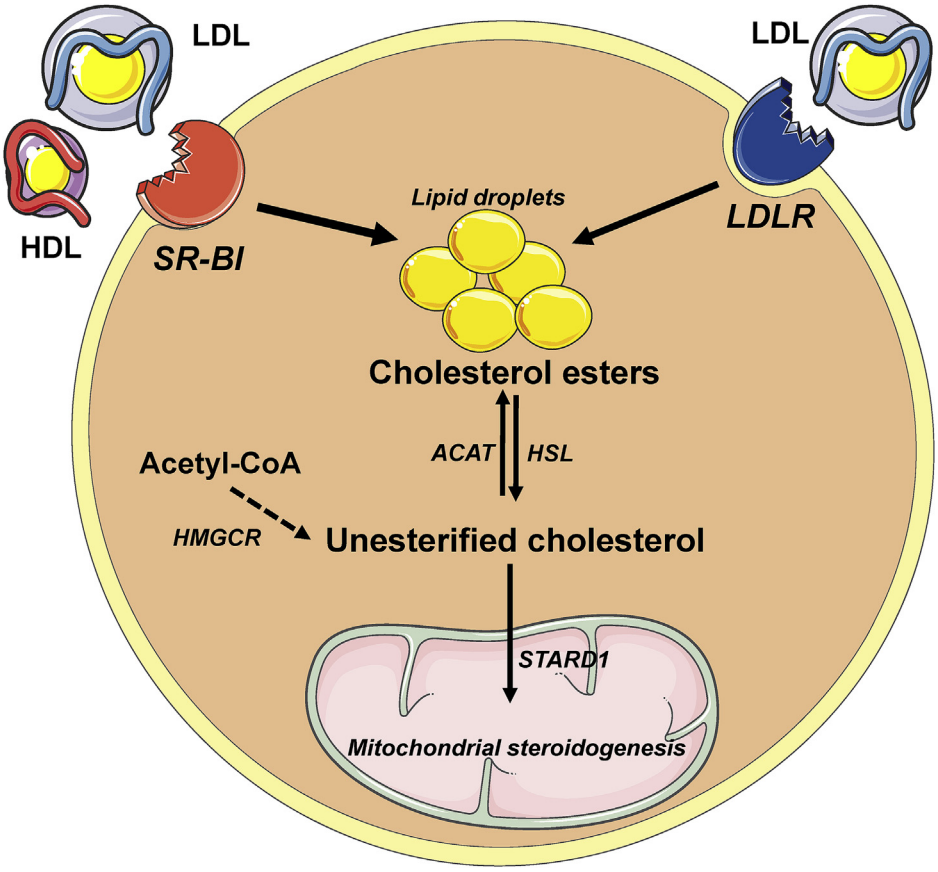

Fig. 3. The sources of cholesterol used by adrenocortical cells to synthesize glucocorticoids. Scavenger receptor BI (SR-BI) and, to a minor extent, the LDL receptor (LDLR) acquire cholesterol esters from circulating lipoproteins that can be stored in lipid droplets or catabolized to generate the unesterified cholesterol pool that is used for steroidogenesis within the mitochondria. In case of a shortage of lipoprotein-derived cholesterol, adrenocortical cells will synthesize the steroidogenic cholesterol substrate from acetyl-CoA. HDL, high-density lipoprotein. LDL, low-density lipoprotein. HMGCR, HMG-CoA reductase, HSL, hormone-sensitive lipase. ACAT, acetyl-CoA acetyltransferase. STARD1, steroidogenic acute regulatory protein.

well as in Cushing's syndrome patients. These proposed studies will not only highlight the potential of SR-BI blocking agents as novel therapy for the treatment of Cushing's disease, but also increase our understanding of SR-BI's role in adrenal glucocorticoid homeostasis in humans.

6. Concluding remarks

It has become evident that glucocorticoids are essential players in the physiological response to stress based upon their ability to modulate both inflammatory and metabolic processes. As a result, disturbances in plasma cortisol levels predispose to the development of a variety of pathologies with atherosclerotic cardiovascular disease being a top killer in patients suffering from primary adrenal insufficiency or Cushing's syndrome/disease. Although the altered glucocorticoid function can in most cases be restored in these different patient groups, i.e. through hormone replacement or steroidogenesis inhibitor treatment, respectively, it should be acknowledged that current therapies do not necessarily reverse the associated susceptibility to cardiovascular disease. In our opinion much attention should therefore be given to the development of a Cushing's disease mouse model that can (1) effectively replicate the effect of hypercortisolemia on atherosclerosis outcome observed in humans and (2) be used to investigate, in a preclinical setting, the relative impact on atherosclerosis susceptibility of already available (e.g. metyrapone) and potentially novel (i.e. SR-BI activity modulators) therapeutic agents that target the adrenal glucocorticoid output.

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