Individuals with metabolic diseases are at a higher risk of developing atherosclerosis, a leading cause of death in the United States and worldwide. Earlier studies have linked dyslipidemia to the initiation and progression of atherosclerosis. However, recent clinical studies raised concerns about the efficacy of lowering plasma cholesterol levels in the progression of atherosclerosis. Although insulin resistance is associated with increased incidence of cardiovascular disease, whether it leads to atherosclerosis independently of its accompanying dyslipidemia remains unclear, largely because of the lack of a suitable animal model to address this question. The CarcinoEmbryonic Antigen-related Cell Adhesion Molecule-1 (CEACAM1) regulates insulin sensitivity by promoting insulin clearance in liver. Accordingly, global null deletion of *Ceacam1* gene impairs hepatic insulin clearance and causes hyperinsulinemia, which in turn, results in **systemic insulin resistance**. Preliminary data show: (*i*) that global *Cc1*$^{-/-}$ null mice develop early atherosclerotic lesions and vascular dysfunction even under normal feeding conditions, and (*ii*) that this occurs in the absence of hyperlipidemia, despite VLDL/LDL cholesterol levels that are usually associated with atherosclerosis regression, not development. This unique animal model of atherogenesis with isolated insulin resistance in the absence of hyperlipidemia demonstrates that systemic insulin resistance resulting from hyperinsulinemia leads to vascular dysfunction and atherosclerosis in the absence of hyperlipidemia. Because phosphorylation of CEACAM1 by both insulin and VEGF receptors regulates Akt1 activation of endothelial Nitric Oxide Synthase (eNOS), an essential step in mediating endothelial function, it is reasonable to **propose that CEACAM1 is the shared downstream element in VEGF and insulin signaling in endothelial cells, whose inactivation impinges upon both pathways and causes endothelial dysfunction in insulin resistance.** To test this hypothesis, the regulatory effect of CEACAM1 on insulin action along the liver/endothelial cell axis will be investigated. **Aim 1** examines whether hyperinsulinemia caused by impaired hepatic insulin clearance, alters insulin action in the endothelial cell, and in this cell-nonautonomous fashion, initiates atheroma development. **Aim 2** examines whether altered signaling through CEACAM1-dependent pathways disrupts the endothelial cell's response to insulin and VEGF, and in this cell-autonomous fashion, drives endothelial dysfunction and initiates atherosclerosis. To investigate the specific role of hepatic and endothelial cell CEACAM1 in the pathogenesis of atherosclerosis and vascular dysfunction, a newly generated set of unique animal models of loss-of-function and gain-of-function will be used. Answering these questions will delineate new CEACAM1-dependent mechanisms underlying atherosclerosis along the liver/endothelial cell axis, and pinpoint sites of pharmacologic intervention.

SPECIFIC AIMS

Individuals with metabolic diseases are at a higher risk of developing atherosclerosis, a leading cause of death in the United States and worldwide. Earlier studies have linked dyslipidemia to the pathogenesis of atherosclerosis. Although the metabolic syndrome (insulin resistance) is associated with increased incidence of cardiovascular disease, whether this abnormality leads to atherosclerosis independently of its accompanying dyslipidemia remains unclear. To investigate this abnormality, several confounding factors should be considered: *(i)* recent studies suggesting that pro-atherogenic dyslipidemia results from enhanced, rather than reduced sensitivity to the actions of insulin–for example, in hepatic VLDL production, *(ii)* the lack of an experimental model to study vascular abnormalities caused by insulin resistance independently of its dyslipidemic effects; and *(iii)* the fact that commonly employed mouse models of atherosclerosis, such as apolipoprotein E-deficient (*ApoE*$^{-/-}$) and LDL receptor-deficient (*LDLr*$^{-/-}$) mice, exhibit a phenotype driven by extremely high levels of plasma cholesterol that can act as the sole atherogenic factor.

The CarcinoEmbryonic Antigen-related Cell Adhesion Molecule-1 (CEACAM1) is a membrane with ubiquitous expression in many cell types, including liver and endothelial cells, but not skeletal muscle and adipose tissue. CEACAM1 regulates insulin sensitivity by promoting insulin clearance in liver. We and others, have shown that mice with global deletion of *Ceacam1* gene (*Cc1*$^{-/-}$) exhibit hyperinsulinemia, resulting from impaired hepatic insulin clearance. Hyperinsulinemia in these mice leads to insulin resistance, increased liver triglyceride content and *de novo* lipogenesis, which drive VLDL-ApoB secretion and triglyceride accumulation in adipose tissue, causing visceral obesity and raising serum fatty acid levels. **Thus, the *Cc1*$^{-/-}$ mouse is a model of hyperinsulinemia causing insulin resistance.**

Preliminary data show: (*i*) that *Cc1*$^{-/-}$ mice with global *Ceacam1* deletion spontaneously develop early atherosclerotic lesions with impaired endothelial-dependent vasodilation under normal feeding conditions, and (*ii*) that this occurs despite VLDL/LDL cholesterol levels that are usually associated with atherosclerosis regression, not initiation. Therefore, *Ceacam1* deficiency causes hyperinsulinemia and insulin resistance, and promotes lipid-independent vascular toxicity. This makes *Cc1*$^{-/-}$ a unique animal model of spontaneous atherosclerosis with isolated insulin resistance in the absence of hyperlipidemia, and provides an *in vivo* demonstration of distinct CEACAM1-dependent mechanisms linking insulin resistance to atherosclerosis.

Insulin and VEGF activation of Akt are required for normal endothelial Nitric Oxide Synthase (eNOS) activity and NO production. In preliminary data, we show that CEACAM1 plays a role in Akt/eNOS activation in response to insulin in endothelial cells, as has been reported for its regulation of VEGF-induced eNOS activity. Thus, we propose the innovative **hypothesis that CEACAM1 is the shared downstream element in VEGF and insulin signaling in endothelial cells, whose inactivation impinges upon both pathways and causes endothelial dysfunction in insulin resistance.** To test this hypothesis, we aim to:

Aim 1: **Examine the pro-atherogenic effects of hepatocyte-specific Ceacam1 deletion. The hypothesis tested in this aim is that insulin resistance caused by impaired hepatic insulin clearance alters endothelial cell function, contributing to atherosclerosis development in a cell-nonautonomous fashion.** Hepatic insulin resistance is pro-atherogenic, as demonstrated by the predisposition to diet-induced atherosclerosis in mice with liver-specific ablation of the insulin receptor. Moreover, preserved hepatic insulin sensitivity can offset the detrimental pro-atherogenic effects of insulin resistance in endothelial cells. To examine the pro-atherogenic effect of hyperinsulinemia caused by impaired hepatic insulin clearance, we will test (*i*) whether conditional deletion of Ceacam1 in hepatocytes is sufficient to cause atherosclerosis, and (*ii*) conversely, whether restoring CEACAM1 exclusively in liver prevents this abnormality in *Cc1*$^{-/-}$ mice.

Aim 2: **Examine cell-autonomous effects of CEACAM1 in endothelial cells that predispose to endothelial dysfunction and atherosclerosis.** Although insulin resistance in vascular endothelial cells is critical to the pathogenesis of atherosclerosis, the mechanism of endothelial cell dysfunction caused by insulin resistance is only partly known. Preliminary data indicate that *Cc1*$^{-/-}$ mice exhibit altered vascular permeability and endothelial dysfunction, as demonstrated by impaired endothelial-dependent vasodilation and reduced NO availability. Ceacam1 knockdown in bovine aortic endothelial cells (bAEC) results in decreased Akt/eNOS activation by insulin and VEGF. This observation leads us **to propose that CEACAM1 acts downstream of insulin and VEGF to regulate Akt/eNOS activation, and that altered signaling through CEACAM1-dependent pathways disrupts the endothelial cell response to insulin and VEGF, driving endothelial dysfunction and initiating atherosclerosis.** To test this hypothesis, we will investigate whether (*i*) conditional deletion of *Ceacam1* gene in endothelial cells is sufficient to cause endothelial dysfunction by eNOS inactivation, and *(ii)* conversely, whether restoring CEACAM1 exclusively in the endothelial cells of *Cc1*$^{-/-}$ mice prevents this abnormality.

A. SIGNIFICANCE

Diabetes and atherosclerosis Patients with type 2 diabetes suffer disproportionately from atherosclerosis and its macrovascular complications (stroke, heart disease) (1). It can be shown that ischemic and non-ischemic heart disease has decreased significantly over the past forty years in the non-diabetic population, whereas in diabetics the decrease has been less significant, and in fact diabetic women have experienced an increase in heart disease (2). Several large clinical outcome studies have cast serious doubts on the role of hyperglycemia as a predisposing factor in the macrovascular complications of diabetes (3-5), and in fact some studies have gone as far as showing that tight glycemic control is associated with increased risk of death from cardiovascular causes (6-8). In addition to these clinical data, animal models indicate that hyperinsulinemia, but not insulin-deficiency, causes endothelial dysfunction (9), and that insulin resistance in liver or in endothelial cells can be sufficient to worsen atherosclerosis in *Ldlr*$^{-/-}$ or *ApoE*$^{-/-}$ knockout mice (10, 11). Nonetheless, insulin resistance is not a monolithic entity, and understanding the intracellular biochemical pathways by which it is brought about remains a highly significant area of research, as it can drive drug development toward specific targets. Accordingly, **the main goal of this grant is to examine the contribution of impaired insulin clearance, an important mechanism of insulin resistance, to vascular dysfunction in the pathogenesis of atherosclerosis.**

Mechanisms of insulin resistance and role of impaired insulin clearance It is a truism that insulin resistance plays a key role in the pathogenesis of type 2 diabetes (12). Although outcome data show that insulin resistance precedes atherosclerosis (13) and trumps hyperglycemia as a risk factor (4), the mechanistic link has not been well established, in part because insulin resistance is genetically, biochemically and pathophysiologically heterogeneous; brought about by different mechanisms in different individuals. Regardless of its initial mechanism, however, insulin resistance is associated with a host of additional abnormalities, key among which are those involving lipid metabolism. It should be remembered that insulin resistance has a self-perpetuating cell biological mechanism. When insulin levels rise in response to impaired insulin responses in peripheral tissues (for example, skeletal muscle), one of the consequences is insulin-induced downregulation of insulin receptors. This, in turn, causes a further impairment of insulin signaling, which increases the demand on the beta cell to make insulin. Moreover, as fewer receptors are available at the cell surface, less insulin gets cleared, because receptor-mediated endocytosis is the main mechanism of insulin clearance from the circulation, mostly in liver. Thus, hyperinsulinemia is often the earliest sign of insulin resistance, and it is caused by a combination of increased secretion and decreased clearance of insulin. **Given that hyperinsulinemia is an independent risk factor for atherosclerosis (13), this proposal builds on the well-established mechanism of hyperinsulinemia caused by impaired hepatic insulin clearance to delineate its mechanistic link to atherosclerosis.**

B. INNOVATION

The role of hyperinsulinemia as a causative factor in insulin resistance has been highlighted by our work, as well as by that of Dr. Richard Bergman. We have shown that, upon insulin binding, the insulin receptor phosphorylates CEACAM1 (Carcinoembryonic antigen-related cell adhesion molecule1), a transmembrane protein, at a single tyrosine residue. This phosphorylation causes binding of the insulin/insulin receptor complex to CEACAM1 and its rapid uptake into clathrin-coated pits, promoting insulin degradation and clearance in the hepatocyte, the main site of insulin clearance. When CEACAM1 phosphorylation is specifically prevented in the hepatocyte by a dominant-negative mutation, insulin internalization and degradation are impaired, and hyperinsulinemia ensues (14, 15). This provided an *in vivo* demonstration that hyperinsulinemia itself, in the absence of peripheral extrahepatic insulin resistance, is sufficient to drive systemic insulin resistance. **Thus, the key innovation of our work is to provide experimental evidence for the causative role of hyperinsulinemia in insulin resistance.** Our hypothesis of a causative role of hyperinsulinemia in insulin resistance has received independent confirmation by the work of Dr. Bergman who showed that an increase in free fatty acid release from adipocytes to the liver impairs insulin clearance, and leads to hyperinsulinemia (16). He has shown, consistent with our work, that hyperinsulinemia can be causative, and not simply a byproduct of insulin resistance. The independent confirmation of our work by a leader in the field provides further evidence of the innovative nature of our work.

In fact, most of the work on insulin resistance focused on examining intracellular pathways of insulin action. This work is very important for understanding the clinical syndrome of insulin resistance. Research on the biochemichal mechanism of insulin action focused on the family of Insulin Receptor Substrates (IRS) and the PI-3-kinase/Akt/Foxo pathway (17). But the discovery of alternative pathways of insulin signaling regulating lipid vs. glucose metabolism in liver (18) has somewhat changed the debate in this field from: how does

insulin signaling regulate lipid synthesis and lipoprotein turnover/secretion?, to: where do the pathways regulating glucose production diverge from those regulating lipid metabolism (19, 20)? The emerging consensus is that Foxo regulates glucose production (along with other factors, such as TORC and FXR), while mTOR regulates Srebp1c-dependent lipogenesis in liver (21). We have shown that acute rise in insulin, as during its pulsatile release from beta cells, decreases the enzymatic activity of Fatty Acid Synthase, a key regulator of hepatic de novo lipogenesis, by inducing CEACAM1 phosphorylation (22). By promoting insulin clearance and regulating FAS activity, CEACAM1 protects insulin sensitivity in liver against the potential lipogenic effect of the high levels of insulin in the portal circulation (23). We should point out that our work neither contradicts nor negates the role of intracellular mechanisms in the pathogenesis of insulin resistance. What we propose is a complementary mechanism that, as highlighted in the section below about human disease relevance, is closely relevant to common forms of insulin resistance in humans.

Human disease relevance*.* The key relevance of our work to human disease is twofold: *(i)* the CEACAM1-dependent mechanism of hyperinsulinemia-induced insulin resistance may be the commonest form of clinical insulin resistance; and *(ii)* CEACAM1 is a potential target for drug development in the metabolic syndrome. With respect to point *(i),* we should emphasize that in most patients with insulin resistance, the safest and most effective interventions are diet and exercise. The shared feature of these two interventions is that they decrease insulin levels, and cause an increase in cell surface insulin receptors, leading to better insulin signaling and increased insulin sensitivity. The role of CEACAM1 in causing hyperinsulinemia has been highlighted by our observations that caloric restriction restores CEACAM1 levels in a genetic rat model of metabolic syndrome (24). In other words, these effective interventions interrupt the vicious cycle of insulin resistance/hyperinsulinemia, and start a virtuous cycle of lower insulinemia/better insulin signaling (25, 26). Thus, we submit that our work addresses what is **arguably the commonest process in the pathogenesis of human insulin resistance: hyperinsulinemia-driven downregulation of insulin signaling.**

With respect to point (ii), one of the challenges faced by the field of insulin resistance is to identify actionable biochemical pathways that can yield drugable targets. Despite the enormous success of the insulin action research community in unraveling mechanisms of insulin action, most of the intracellular mediators of insulin signaling are poor drug targets, either by virtue of their biochemical structure (e.g., IRS proteins) or because they are shared in common with other key cell biological processes (e.g., Akt, mTOR) or both (Foxo). Because CEACAM1 is a cell surface protein with a large extracellular domain, it may make a better target for drug development. We realize that this is simply speculation at this point, but the urgency of developing better remedies against insulin resistance provides a strong rationale to pursue the proposed research.

Atherosclerosis remains arguably the critical unsolved issue in diabetes treatment. Not only do the macrovascular complications account for much of the excess mortality related to diabetes, but they account for up to 50% of heath care cost related to it (27). Whereas existing therapeutics, while far from ideal, can be effective in lowering plasma glucose levels and therefore controlling microvascular complications, such as eye disease, neuropathy, and nephropathy, the dyslipidemia of diabetes represents an unmet need for treatment. The lipid triad of insulin resistance consists of increased VLDL-triglyceride, increase small dense LDL, and decreased HDL. Current hypolipidemic agents are not very effective against this triple threat. We submit that only by better understanding the genetic, biochemical, and integrated physiological mechanisms of the effects of insulin resistance on atherosclerosis we can make progress toward its treatment. The proposed research is unique, in that relatively few investigators have focused on this specific aspect of the problem and is innovative, in that it pursues a mechanism that departs from the main approach to this problem (intracellular signaling). We envision that the studies proposed in this application will reveal new aspects of the metabolic syndrome, and of the interactions between insulin sensitivity in hepatic and endothelial cells, thereby adding a new dimension to experimental therapeutics in this area.

C. APPROACH

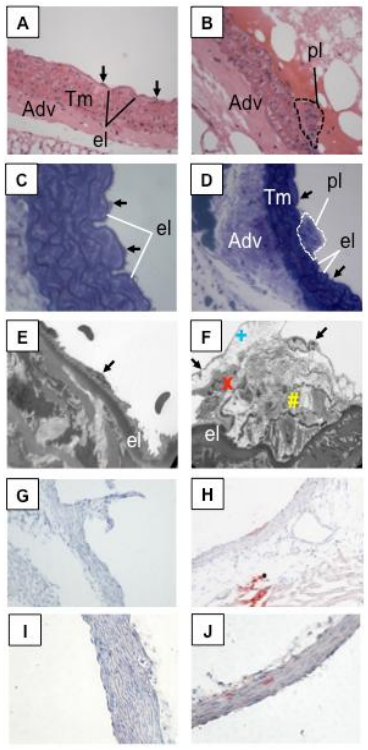
C1. Aim 1: Examine the pro-atherogenic effects of hepatocyte-specific Ceacam1 deletion.

Hypothesis: **Insulin resistance caused by impaired hepatic insulin clearance alters endothelial cell function, contributing to atherosclerosis development in a cell-nonautonomous fashion.**

C1.a. Impact: Earlier studies have linked dyslipidemia to the initiation and progression of atherosclerosis (28-31). However, recent clinical studies show atherosclerotic plaques progression despite lowering plasma cholesterol levels (32). Thus, it is incumbent upon us to identify mechanisms linking metabolic to vascular abnormalities with an eye to develop a suitable therapeutic strategy in patients with metabolic syndrome. Although outcome data show that insulin resistance (and hyperinsulinemia) precedes atherosclerosis (13)

and trumps hyperglycemia as a risk factor (5), it remains unclear whether this metabolic abnormality leads to atherosclerosis independently of its accompanying dyslipidemia, in part due to the lack of an animal model to study vascular effects of hyperinsulinemia in a non-hyperlipidemic milieu (33). Preliminary data indicate that the ***Cc1*$^{-/-}$ mouse is a unique model of atherogenesis with isolated insulin resistance in the absence of hyperlipidemia.** The similarity in the metabolic phenotype between this mouse and that of L-SACC1 mouse with liver-specific inactivation of Ceacam1 demonstrates that insulin resistance in *Cc1*$^{-/-}$ mice is caused by hyperinsulinemia resulting from impaired hepatic insulin clearance due to lack of CEACAM1 in hepatocytes (15, 34, 35). Thus, we set out to examine the role of liver CEACAM1 in the pathogenesis of atherosclerosis. **This will determine whether hyperinsulinemia is the inciting factor in systemic insulin resistance that promotes atherogenesis, as reported in some outcome studies (13).** Answering these questions would pinpoint sites of pharmacologic intervention.

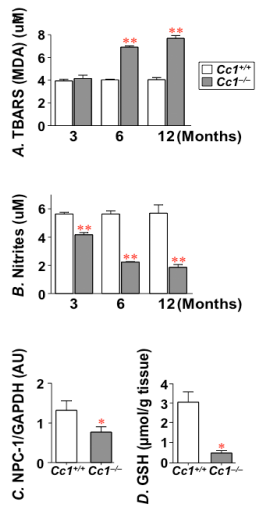
Fig. 1:



C.1b. Preliminary Data: Mutant *Ceacam1* mice exhibit several risk factors of atherosclerosis, including a cluster of metabolic abnormalities (insulin resistance, visceral obesity and hepatic steatosis) (14, 15, 34, 35), inflammation (36), defective vascular remodelling (37), and increased endothelial permeability (38). In preliminary data described below, we demonstrate that **global loss of *Ceacam1* causes endothelial dysfunction and spontaneous development of early atherosclerotic lesions on a chow diet. Thus, loss of *Ceacam1* gene illustrates vascular dysfunction associated with systemic insulin resistance.**

***Spontaneous development of pre-atherosclerotic lesions in the aorta of Cc1*$^{-/-}$ *mice* –** Histological analyses by H&E and semi-thin sections by Toluidine blue revealed early atherosclerotic plaques in the aorta (ascending aorta and aortic arch) of 6 month-old male *Cc1*$^{-/-}$ mice (Fig. 1 A-B; dotted line, plaque: pl; magnification: 400x). High-power light microscopic analysis on semi-thin sections (1 µm) showed plaques in the aortic intima of *Cc1*$^{-/-}$ mice (Fig. 1 C-D; Black arrows: endothelial cells, Adv: Adventitia, el: inner elastic membrane, Tm: Tunica media; 400x for D, 1000x for C). Electron microscopic analysis on ultrathin sections (80 nm) detected atherosclerotic plaques with fibrotic deposition, cellular debris and lipid accumulation in the sub-endothelial space, and destruction of the inner elastic membrane in the intima of *Cc1*$^{-/-}$ aorta (Fig. 1 E-F; x: Smooth muscle cells; +: Sub-endothelial edema; #: Lipid depositions; 3000x for E and F). Accumulation of mast cells within the aortic adventitia opposite to the plaque indicated activation of inflammatory processes within the aortic wall of *Cc1*$^{-/-}$ mice (not shown). Oil Red-O (Fig. 1 G-H; 200x) and MOMA-2 (Fig. 1 I-J; 400x) staining confirmed macrophage infiltration in the vessel wall and lipid deposition in plaques, respectively.

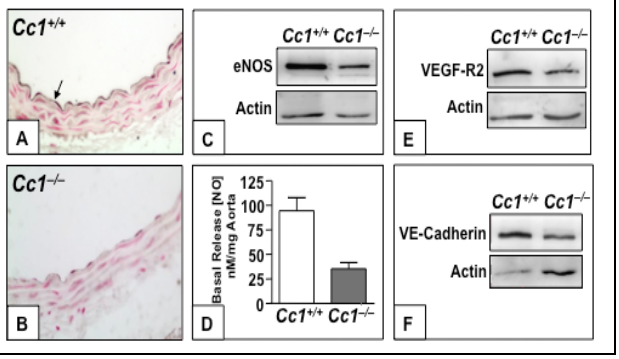
Fig. 2:



* *p<0.01*, ** *p<0.001 vs. Cc1*$^{+/+}$*;(n ≥ 6)*

***Oxidative stress and endothelial dysfunction in Cc1*$^{-/-}$ *mice* –** Consistent with increased lipid peroxidation with the development of inflammatory vascular damage and atherosclerosis (39), serum TBARS concentration was highly elevated in *Cc1*$^{-/-}$ mice starting at 6 months of age (Fig. 2A). In addition to increased inflammatory cytokines and reactive oxygen species (ROS) in arterial walls, decreased level and/or activity of endothelial nitric oxide synthase (eNOS) causes oxidative stress and nitroso-redox imbalance (40, 41). Accordingly, immunohistochemical analysis showed a significant reduction in eNOS staining in the endothelial cell layer of aortas of *Cc1*$^{-/-}$ mice (Fig. 3A vs 3B, arrow).

Fig. 3



Of note, the endothelial layer was identified by co-staining with CD31 (not shown). Western analysis also demonstrated a ~40% decrease in eNOS protein levels in aortic lysates, when normalized against β-actin (Fig. 3C). Consistently, basal level of released nitric oxide (NO) (nM/mg aorta) was reduced by ~60% in aortic segments isolated from 6 month-old *Cc1*$^{-/-}$ (Fig. 3D), and the level of serum nitrite, a nitric oxide oxidation product, was significantly decreased in *Cc1*$^{-/-}$ even at 3 months of age (Fig. 2B), suggesting **reduced basal eNOS activity and NO bioavailability in the aorta of *Cc1*$^{-/-}$ mice**. Because impaired endothelial - dependent vasodilation, a key initial step in atherosclerosis (42), is mediated by reduced NO bioavailability (43), the data demonstrate endothelial dysfunction in *Cc1*$^{-/-}$ mice (44, 45).

Table 1:
Metabolic Parameters

	Cc1 $^{+/+}$	*Cc1* $^{-/-}$
Total Cholesterol (mg/dl)	50 ± 3.8	66 ± 4.0 **
VLDL (mg/dl)	3.0 ± 0.7	6.0 ± 0.9 *
HDL (mg/dl)	41 ± 3.3	45 ± 3.3
LDL (mg/dl)	13 ± 0.8	16 ± 1.0 **
FFA (mEq/l)	0.56 ± 0.02	0.97 ± 0.08 *
Triglyceride (TG) (mg/dl)	50.9 ± 3.25	44.8 ± 4.00
Hepatic TG (µg/mg prot)	58.7 ± 4.23	91.3 ± 6.32 *

Lipid profile in 6 month-old mice (n > 6)
* *P* <0.05 and ** *P* <0.01 vs. *Cc1*$^{+/+}$.
Values are expressed as mean ± SEM

Absence of significant dyslipidemia in Ceacam1 knockouts – As previously shown (34), plasma triglyceride is normal in *Cc1*$^{-/-}$ mice despite increased hepatic triglyceride production and secretion, owing to redistribution to white adipose tissue (WAT), as supported by increased serum free fatty acids (FFA) output (Table 1). Wild-type mice are resistant to atherosclerosis even when placed on a high fat, but non-cholic acid containing diet, owing to their relatively low levels of circulating ApoB-containing lipoproteins. *Cc1*$^{-/-}$ mice, unlike genetic models of atherosclerosis such as *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$, have low cholesterol levels and human-like high-density lipoprotein (HDL) cholesterol (Table 1). Although non-HDL cholesterol in *Cc1*$^{-/-}$ mice was slightly greater than that found in controls and serum ApoB 48/100 level is elevated (34), **VLDL/LDL cholesterol was at levels usually associated with regression rather than initiation of lesions (46).**

Elevated inflammatory state in Cc1*$^{-/-}$ *mice – Another important factor in atherosclerosis progression is chronic inflammation of the arterial wall (33, 47). Quantitative real-time RT-PCR (qRT-PCR) analyses revealed increased mRNA levels of F4/80 (a macrophage marker), T cells (CD3+, CD4+, and CD8+T), and TNFα inflammatory cytokine in *Cc1*$^{-/-}$ aortas (Table 2). mRNA of **VCAM-1 was also elevated by ~2-fold** in the aorta of *Cc1*$^{-/-}$ mice (Table 2), supporting the initiation of atherosclerotic lesions (48, 49) and suggesting increased transmigration of circulating leukocytes to the sub-endothelial space (50). The increase in VCAM-1 mRNA could be due to reduction in eNOS (51) and to increased cytokine levels, including TNFα and IL6 (52, 53). Moreover, mRNA levels of toll-like receptor-2 and -4 (TLR-2 and -4) were ~2-fold higher in the aorta of *Cc1*$^{-/-}$ than *Cc1*$^{+/+}$ mice (Table 2), perhaps in response to high serum FFA levelss (54). Elevated mRNA levels of IL-6 and TNFα, transcriptional target genes of TLR signalling, points to activation of TLR-2 and -4, and initiation of inflammatory pathways in the aorta of these mice (55). Moreover, *Cc1*$^{-/-}$ mice also exhibit increased inflammation in adipose tissue, consistent with visceral obesity. mRNA levels of WAT-associated F4/80 and the proinflammatory IL-6 and TNFα cytokines, in addition to monocyte chemotactic protein-1 (MCP-1) and interferon (IFN)-γ chemokines were elevated in *Cc1*$^{-/-}$ mice (by > 2-fold). Thus, *Ceacam1* deletion elevates the overall inflammatory state.

Table 2:

	Cc1 $^{+/+}$	*Cc1* $^{-/-}$
F4/80	0.04 ± 0.01	0.11 ± 0.01 *
TNFα	1.08 ± 0.31	4.78 ± 0.36 *
VCAM-1	0.03 ± 0.01	0.06 ± 0.01 *
CD3	0.02 ± 0.004	0.99 ± 0.006 **
CD4	0.01 ± 0.01	0.23 ± 0.01 **
CD8	0.02 ± 0.010	0.38 ± 0.004 **
TLR2	0.53 ± 0.06	1.07 ± 0.11 **
TLR4	0.15 ± 0.01	0.28 ± 0.04 *

mRNA levels of inflammatory markers in aorta of 6 month-old male mice
*$p < 0.01$ vs. *Cc1*$^{+/+}$, **$p < 0.001$ vs. *Cc1*$^{+/+}$ ($n > 6$

Depleted GSH and decreased NPC-1 in the vasculature of Cc1*$^{-/-}$ *mice – Reduction in Niemann–Pick type C1 protein (NPC1) causes depression of glutathione (GSH), a main mitochondrial defense system against the cytotoxic effect of TNFα, and accumulation of the cytotoxic free cholesterol in macrophages, an essential event in the development of atherosclerosis (56). qRT-PCR analysis revealed a significant (~6-fold) reduction in NPC1 mRNA levels of aortic tissue of *Cc1*$^{-/-}$ relative to *Cc1*$^{+/+}$ mice (Fig. 2C). This was associated with a marked reduction in the GSH level in aortic tissue from 6 month-old *Cc1*$^{-/-}$ mice (Fig. 2D). *Of note,* TNF*α* could cause a robust cytotoxic effect in light of the absence of the counter-regulatory anti-inflammatory effect of CEACAM1 in T-cells (36) and reduced GSH-mediated defense.

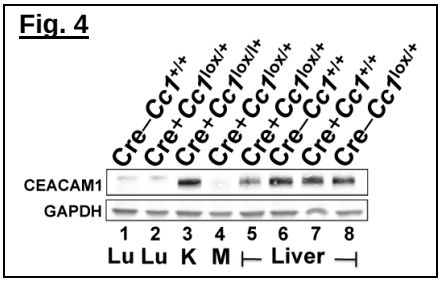
C.c. Rationale: *Hepatic insulin resistance is pro-atherogenic,* as demonstrated by the predisposition to diet-induced atherosclerosis in LIRKO mice with liver-specific ablation of the insulin receptor (10), and by the partial protection from atherosclerosis in mice with preserved hepatic insulin sensitivity in the face of peripheral insulin resistance in muscle, fat and endothelial cells (31). Global null mutation of *Ceacam1* causes systemic insulin resistance by impairing hepatic insulin clearance and causing hyperinsulinemia (34).

With the onset of insulin resistance at 6 months of age, *Cc1*$^{-/-}$ mice develop atherosclerotic plaques in the absence of dyslipidemia (preliminary data). **Therefore, *Ceacam1* deficiency, acting presumably through hyperinsulinemia and systemic insulin resistance, promotes a state of lipid-independent vascular dysfunction that is conducive to the initiation of atherosclerotic lesions.**

How do changes in plasma insulin and insulin resistance promote vascular disease? Hyperinsulinemia causes endothelial dysfunction by impairing Akt1/eNOS activity in endothelial cells (9), among other things. In preliminary data we show that eNOS protein level is reduced in the aorta of *Cc1*$^{-/-}$ mice in parallel to decreased NO production in aortic segments. But SiRNA-mediated knockdown of Ceacam1 in bAEC cells did not alter eNOS levels [(38) and in preliminary data], indicating that reduced aortic eNOS content in *Cc1*$^{-/-}$ mice is primarily caused by systemic insulin resistance that causes insulin resistance in endothelial cells [by downregulating insulin receptors- (57)] and reduces eNOS expression via a Foxo1-mediated mechanism (57, 58). Because loss of InsR in endothelial cells (11) predisposes to atherosclerosis, **we postulate that increased atherosclerosis in *Cc1*$^{-/-}$ mice is caused by sustained hyperinsulinemia causing endothelial cell insulin resistance, which in turn, leads to endothelial dysfunction and atherogenesis.** Thus, it is likely that endothelial dysfunction in *Cc1*$^{-/-}$ mice is caused by a cell-nonautonomous mechanism, giving rise to defective eNOS level (and activity) as the primary vascular consequences of sustained hyperinsulinemia. We realize that insulin resistance in other cells, such as macrophages, likely contributes to atherosclerosis in *Cc1*$^{-/-}$ mice, but as the effect of macrophage InsR on this process is controversial and not very marked (59-61), we will omit it from further consideration. Thus, we will simplify the discussion focusing on the effect of hyperinsulinemia on insulin action along the liver/endothelial cell axis.

C.1d. Proposed experiments: To test whether CEACAM1's regulation of hepatic insulin clearance and systemic insulin sensitivity affect atheroma formation by modulating insulin action in endothelial cells, we will test whether conditional deletion of *Ceacam1* gene in hepatocytes is sufficient to cause atherosclerosis, and conversely, whether restoring CEACAM1 exclusively in liver of *Cc1*$^{-/-}$ mice prevents this abnormality. The purpose of this aim is to investigate whether **hepatic insulin resistance is causative, permissive or synergistic with endothelial dysfunction to cause atherosclerosis**. To simplify the discussion of this aim, we will describe a model that is well suited to **dissect the liver/endothelial insulin resistance interaction**.

i. Generation of the experimental models:



The liver-specific AlbCc1*$^{fl/fl}$ *null mouse – We have obtained the floxed *Cc1*$^{loxP/loxP}$mouse (on the B6/129sv genetic background) from Dr. Sue-Hwa Lin at MD Anderson. The conditional targeting construct inserted a loxP-neo cassette in intron 6 and a loxP fragment in intron 9, thus deleting a sequence that encodes the cytoplasmic domain that is required for phosphorylation of CEACAM1 and its regulation of insulin acton (14). In collaboration with Dr. Richard Blumberg (Brigham and Women's Hospital), we have successfully used this floxed *Cc1*$^{loxp/loxp}$ mouse to generate a conditional T cell-specific null *Ceacam1* mouse (36). We followed the same strategy to generate the conditional liver-specific null *Ceacam1* mouse. Briefly, we crossed *Cc1*$^{loxp/loxp}$ mice with transgenic mice expressing Cre under the transcriptional control of the albumin gene promoter (Albumin-Cre) propagated on the C57BL/6J background (Jackson Laboratories). Genotyping by qRt-PCR using primer sets from Cre, and floxed and wild-type *Ceacam1,* identified heterozygous mice with one floxed Ceacam1 allele with or without Cre. Heterozygous mice have been backcrossed 5 times with C57BL/6 (BL6) mice. Tissue-specific deletion of CEACAM1 from liver has been confirmed by Western analysis using an antibody with an antigenic determinant against the extracellular domain (Fig. 4). Relative to controls (lane 6-8), heterozygous mice for floxed Ceacam1, lost 50% of their CEACAM1 content in liver, in the presence of Cre (lane 5). In contrast, CEACAM1 expression in lungs (Lu) (lane 2 vs 1), kidney (K, lane 3) and skeletal muscle (M, lane 4) were unaffected [CEACAM1 protein expression in sk. muscle is not significant (14, 62, 63)]. We will breed to homozygosity and use male mice homozygous for *Ceacam1* floxed allele and bearing Cre (AlbCre+*Cc1*$^{loxp/loxp}$) [for simplicity termed: **Alb*Cc1*$^{fl/fl}$**]. As controls, we will use homozygotes with wild-type *Ceacam1* allele with Cre (AlbCre+*Cc1*$^{+/+}$) [for simplicity termed: **AlbCre**] and homozygotes with *Ceacam1*-floxed allele, but without Cre (AlbCre –*Cc1*$^{loxp/loxp}$) [***Cc1*$^{lox/lox}$**], all from the same breeding to rule out potential confounding effects brought about by floxing and introducing Cre.

The liver-specific rescue Cc1*$^{-/-xliver+}$ *mouse – We initially generated L-CC1 mice overexpressing rat CEACAM1 in liver, using ApolipoproteinA1 (ApoA1) promoter, as in L-SACC1 mice bearing the S503A Ceacam1 mutant (15). This promoter drove liver-specific expression, as assessed by absence in intestine,

the other main site of ApoA1 production (64), and in other tissues (not shown). We then bred *Cc1*$^{-/-}$ with L-CC1 to obtain heterozygous mice for the Cc1 locus, which were subsequently backcrossed with BL6 six times to obtain a progeny of *Cc1*$^{+/-}$ with or without the transgene. Intercrossing this progeny produced *Cc1*$^{-/-}$ with the transgene (***Cc1***$^{-/-xliver+}$) and their control littermates: *Cc1*$^{-/-}$ without the transgene (for simplicity, ***Cc1***$^{-/-}$), *Cc1*$^{+/+}$ without the transgene (***Cc1***$^{+/+}$**)** and *Cc1*$^{+/+}$ with the transgene (**L-CC1**). Western analysis revealed absence of endogenous mouse CC1 (mCC1) in all tissues of ***Cc1***$^{-/-xliver+}$ mice, which express the rat CEACAM1 (rCC1) **transgene only in liver** (Liv), but not kidney (K) or intestine (Int) (Fig. 5).

Fig. 5:

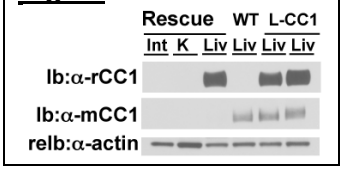


Table 3:

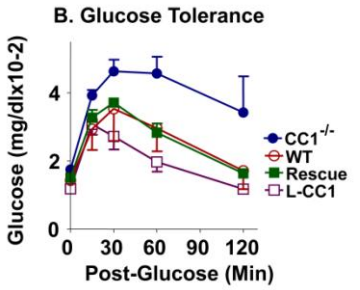
Metabolic Parameters

	Cc1$^{+/+}$	*Cc1*$^{-/-}$	*Cc1*$^{-/-xliver+}$
Body weight (g)	24.1 ± 0.30	25.5 ± 0.23 ^{A}	24.3 ± 0.31
Serum insulin (ng/ml)	0.47 ± 0.04	0.82 ± 0.04 ^{A}	0.50 ± 0.08
Serum C-peptide (ng/ml)	1.02 ± 0.50	2.32 ± 0.41 ^{A}	0.94 ± 0.11
Steady state C/I ratio	4.01 ± 0.20	2.41 ± 0.12 ^{A}	4.48 ± 0.11
Serum FFA (mEq/l)	0.63 ± 0.03	0.80 ± 0.03 ^{A}	0.70 ± 0.06
Serum TG (mg/dl)	56.2 ± 2.24	71.2 ± 3.00 ^{A}	54.4 ± 2.46
Fasting glucose (mg/dl)	113. ± 1.42	114. ± 0.86	115. ± 0.72
Random glucose (mg/dl)	125. ± 6.43	124. ± 3.05	130. ± 1.22

Male mice (n>8 per group) were phenotyped at 2 months of age.
^{A}***P*<0.05 *Cc1*$^{-/-}$ *vs* other groups of mice.**

L-CC1 mice and *Cc*$^{+/+}$ littermates were used as controls. Because they share a similar phenotype, we only include the metabolic parameters of *Cc*$^{+/+}$. Preliminary data on 2 month-old male mice propagated on ~98% BL6, show that *Cc1*$^{-/-}$ mice exhibit higher body mass, hyperinsulinemia and impaired insulin clearance (as measured by C-peptide/Insulin ratio at steady state) (Table 3, *Cc1*$^{-/-}$ vs *Cc1*$^{+/+}$ mice). As previously reported (34), serum triglyceride is elevated in *Cc1*$^{-/-}$ mice at 2 months of age (but declines thereafter). Rescuing Ceacam1 expression specifically in the liver reversed these metabolic abnormalities (Table 3, *Cc1*$^{-/-xliver+}$ vs. *Cc1*$^{-/-}$ mice). Metabolic rescuing persists, as indicated by failure of 8 month-old *Cc1*$^{-/-xliver+}$ mice to gain more body weight than WT mice (not shown) and to develop glucose intolerance like their *Cc1*$^{-/-}$ counterparts (Fig. 6). Although further analysis is required, these data confirm: **1) that the principal role for CEACAM1 in liver is to promote hepatic insulin clearance, and 2) that insulin clearance plays a key role in the pathogenesis of insulin resistance.**

Fig. 6:



ii. Phenotyping:

Metabolic profile – To characterize the metabolic phenotype of these mice, we will evaluate body weight, fat distribution (by NMR), serum glucose, insulin and C-peptide levels (15, 34, 65-67). We will measure insulin clearance by tail vein [I^{125}] Insulin injection and [I^{125}] Insulin degradation in primary hepatocytes. For insulin action, we will perform insulin and glucose tolerance tests, analyze insulin action in primary hepatocytes, and employ the hyperinsulinemic-euglycemic clamp analysis to determine insulin action in liver, skeletal muscle, and white and brown adipose tissue (In collaboration with Jason Kim at the Univ. of Mass). In collaboration with Ira Goldberg at Columbia University (Cf letter), we will assess total serum triglyceride and cholesterol levels, and their lipoprotein fractions by FPLC fractionation (as in preliminary data). We will determine ApoB48/100 in serum and lipid metabolism in liver [tissue triglyceride levels; fatty acid synthase activity and cholesterol synthesis in light of the fact that CEACAM1 exerts a negative effect on these parameters (22, 66); VLDL secretion by the Triton WR1339 Method (68, 69) and fatty acid oxidation (^{3}H Palmitate) (67)]. We will also assess macrophage recruitment to white adipose tissue and the mRNA levels of its associated cytokines.

We will perform longitudinal studies on 10 male mice at 3, 6 and 9 months of age. We reached this number based on data on the global *Cc1*$^{-/-}$ null mouse (34) and using nQuery Advisor version 6.0 software program, using the formula for repeated measure analysis of variance (ANOVA) for the metabolic profile to test our hypothesis at 0.05 level of significance with at least 80% power.

Anticipated Results: We expect **Alb*Cc1*$^{fl/fl}$ null mice to phenocopy the altered metabolic phenotype of L-SACC1** with liver-specific inactivation of CEACAM1 (15). Moreover, because they are propagated onto the BL6 background, we expect all metabolic abnormalities regarding insulin and lipid metabolism to precede the development of overt insulin resistance, as we have shown in the global *Cc1*$^{-/-}$ mouse (34). The phenotype includes hyperinsulinemia due to impaired insulin clearance; an increase in hepatic triglyceride production and redistribution to the white adipose tissue (WAT) to cause visceral obesity, which in turn, leads to FFA mobilization and macrophage recruitment to WAT and release of pro-

inflammatory adipokines, all of which can promote endothelial dysfunction and atherosclerosis (70-73). Should vascular abnormalities be observed in Alb*Cc1*$^{fl/fl}$, as in *Cc1*$^{-/-}$ mice together with increased development of inflammatory pathways in the aorta (see below), we would assign a primary role for hepatic insulin resistance and altered lipid metabolism in this process.

We also expect to observe a moderate increase in serum VLDL-ApoB, VLDL and LDL cholesterol levels with a VLDL/LDL cholesterol level that is not usually associated with initiation of lesions (46). The mechanism of increased non-HDL cholesterol level may be related to elevated hepatic cholesterol synthesis, as observed in L-SACC1 (66) and *Cc1*$^{-/-}$ (35) mice. Moreover, the increase in VLDL production may lead to greater LDL production. Alternatively, hepatic insulin resistance, as seen in the liver-specific insulin receptor knockout mouse (LIRKO), could lead to reduced LDL clearance (10).

The metabolic phenotype of ***Cc1***$^{-/-xliver+}$ **is expected to be the opposite to that of Alb*Cc1*$^{fl/fl}$ null mice**, based on their normal metabolic profile (Table 3, Fig. 6). In light of the insulin sensitizing effect of CEACAM1 and its inhibitory effect on fatty acid and cholesterol synthesis (22, 66), VLDL production and ApoB levels are expected to be lower in *Cc1*$^{-/-xliver+}$ than *Cc1*$^{-/-}$ mice. Hence, restoration of CEACAM1 in hepatocytes is expected to prevent triglyceride redistribution to WAT, visceral obesity, lipolysis and adipokine release.

Vascular phenotyping – As in preliminary data, we will: (*i*) Assess **plaque formation** by H&E and Toluidine blue staining, and electron microscopic analyses of the aorta (ascending aorta and aortic arch); (*ii*) Determine the size and cellular composition of lesions; (*iii*) Quantify markers of **cell adhesion** (ICAM, VCAM1) that affect leukocytes' adherence to the endothelium; (*iv*) Quantify markers of **inflammation** in the arterial wall: we will determine macrophage infiltration by MOMA-2 staining and quantifying mRNA levels of TLR-2 and TLR-4, and of several inflammatory markers (cd68 and others per preliminary data); and (*v*) Assess **apoptosis** (Tunnel staining and c-caspase 3 cleavage) and cell viability and proliferation in isolated MLEC and monocytes to investigate whether apoptosis occurs in endothelial cells (as part of early lesions) (74, 75) and rule out macrophage apoptosis since this occurs in advanced lesions (76, 77).

We will examine **lipid deposition** in the vessel wall by Oil Red-O staining and by quantitatively extracting lipids from the brachiocephalic artery with chloroform/methanol. We will also stain the whole aorta with Sudan IV to visualize atherosclerotic plaques in flat preparations (en face preparations).

We will assess **oxidative stress** and **nitroso-redox imbalance** [serum lipid peroxidation by **TBARS**; **GSH** and NPC1 levels in the vasculature; **ROS generation** in MLEC in response to H_2O_2, free cholesterol, and 7-keto-cholesterol, and **NADPH oxidases** mRNA and enzymatic activity (21)]. We will examine **endothelial dysfunction** [**eNOS content** by immunohistochemical and Western analyses and **activation** (by phosphorylation) in the arterial wall, basal level of NO in aortic segments and the level of serum nitrite, a nitric oxide oxidation product].

To evaluate the functional consequences of altered NO production, we will measure: 1) **Ach-induced vascular relaxation** in phenylephrine precontracted **aortic rings**, since this depends mostly on endothelium-derived NO; and 2) ***in vivo*** **Leukocyte-Endothelial Cell Interaction,** using intravital microscopy of the mesenteric circulation after labeling of leukocytes with a fluorescent dye, since decreased eNOS activity reduces the interaction of monocytes with endothelium. To delineate the effect of CEACAM1 in hematopoietic cells in leukocyte-endothelium interaction, we will perform **Mononuclear Cell Transfer** experiments by intravenously injecting fluorescence-tagged peripheral blood mononuclear cells from *Cc1*$^{-/-}$, Alb*Cc1*$^{fl/fl}$, *Cc1*$^{-/-xliver+}$ mice and their controls into wild-type recipients and examining leukocyte-interaction with the endothelium. These experiments will be performed at the laboratory of our collaborator, Dr. Scalia (Cf. letter).

To assess whether and how leukocytes with no CEACAM1 could affect plaque development differently from leukocytes with normal expression of CEACAM1, we will perform **bone marrow transplantation** from control into *Cc1*$^{-/-}$ and Alb*Cc1*$^{fl/fl}$ mice. To further address potential synergy between the pro-atherogenic effect of monocytic and hepatocytic CEACAM1, we will transfer bone marrow from global *Cc1*$^{-/-}$, *Cc1*$^{-/-xliver+}$ and their control mice into Alb*Cc1*$^{fl/fl}$ recipients. Dr. Stanislaw Stepkowski, an intramural expert immunologist and transplantation scientist, will collaborate with us on these experiments (Cf letter).

To measure **insulin action in the vasculature**, we will intravenously inject fasted mice with insulin (78) to: *(i)* examine insulin signaling pathways, including the phosphorylation of InsRβ, Akt1, ERK1/2, foxo1, CEACAM1 and eNOS (by Western analysis) in aorta, femoral arteries and lung, since endothelial cells constitute 50% of cellular populations in this tissue. We will also examine insulin action in murine lungs endothelial cells (MLEC) derived from these mice. Because VEGF signaling in endothelial cells affects atheroma formation

(79, 80), we also plan to examine VEGF signaling in aortic lysates in the presence of ATP (81) and in isolated MLEC cells. (We will train on isolating MLEC at the laboratory of Dr. Beauchemin-Cf letter).

Anticipated Results: We expect that sustained hyperinsulinemia in Alb*Cc1*$^{fl/fl}$ null exerts a pro-atherogenic effect by causing insulin resistance in endothelial cells. Conversely, rescuing CEACAM1 in liver is expected to mitigate the development of atherosclerotic plaques in *Cc1*$^{-/-xliver+}$ by normalizing insulin sensitivity (preliminary data). This prediction is supported by the demonstration of a partial protection from atherosclerosis in mice with preserved hepatic insulin sensitivity in the face of peripheral insulin resistance in muscle, fat and endothelial cells (31).

Activation of InsR and Akt1, the dominant vascular isoform (82), is required for normal eNOS activity (11, 83) and NO production in endothelial cells in response to insulin (84). By downregulating insulin receptors (57), **hyperinsulinemia** in Alb*Cc1*$^{fl/fl}$ mice is expected to cause insulin resistance in the endothelial cells, manifested in part by a defect in InsR/Akt activation (phosphorylation) and subsequent reduction in eNOS transcription via a Foxo1-mediated mechanism (57, 58). Together with the expected decrease in VEGF levels via inactivating the InsR/Akt pathway (85), this is expected to decrease eNOS phosphorylation in response to insulin and VEGF in isolated endothelial cells from Alb*Cc1*$^{fl/fl}$. We also expect decreased NAPDH oxidase activity with increased ROS generation and activation of proinflammatory pathways in response to cholesterol loading in endothelial cells from these mice. Thus, Alb*Cc1*$^{fl/fl}$ null mice will exhibit an increase in oxidative stress (and a nitroso-redox imbalance) with a decrease in endothelial function, eNOS activation and NO bioavailability (43), as will be further assessed by impaired endothelial-dependent vasodilation, one of the initial key steps of atherosclerosis (42).

Moreover, reduction of eNOS activity causes atheroma formation, in part by increasing the interaction of monocytes and other leukocytes with endothelium. To test this hypothesis, we will perform in vitro monocyte rolling and adhesion assays using primary endothelial cells cultured from *Ceacam1* knockouts. We expect that the number of firmly adhering leukocytes will be higher in both *Ceacam1* deletion mutants (*Cc1*$^{-/-}$ and Alb*Cc1*$^{fl/fl}$ mice) than in controls and *Cc1*$^{-/-xliver+}$ rescue mice, despite normal plasma lipids, as in mice with InsR deletion from the endothelial cells (11). Given that initiation of atherosclerotic lesions is associated with increased endothelial expression of VCAM-1 (but not ICAM1) (48, 49), and that this allows the transmigration of circulating leukocytes to the sub-endothelial space (50), we expect increased levels of VCAM1 in lung and MLEC of Alb*Cc1*$^{fl/fl}$, as in *Cc1*$^{-/-}$ mice (preliminary data). The increase in VCAM1 could be due to reduction in eNOS (51); to increase in cytokine levels, including TNFα and IL6 (preliminary data) (52, 53), and to reduced insulin action (11) and associated increase in Foxo1 nuclear localization and activation (86).

The contribution of CEACAM1 in hematopoietic cells to leukocyte-endothelium interaction will be assessed in **mononuclear cell transfer** from *Cc1*$^{-/-}$, Alb*Cc1*$^{fl/fl}$, *Cc1*$^{-/-xliver+}$ and their controls into wild-type recipients. A higher number in firmly adhering leukocytes from *Cc1*$^{-/-}$ relative to Alb*Cc1*$^{fl/fl}$ mice would indicate a role for monocytic CEACAM1 in the vascular phenotype. If the number is the same between deletion mutants or *Cc1*$^{-/-xliver+}$, we would exclude a significant role for monocyte CEACAM1 in atheroma formation.

The role of CEACAM1 in monocytes (and macrophages) has not been well delineated, but macrophages lacking CEACAM1 (as in *Cc1*$^{-/-}$ and *Cc1*$^{-/-xliver+}$) could affect plaque development by a variety of mechanisms. If bone marrow-derived cells from global *Cc1*$^{-/-}$or *Cc1*$^{-/-xliver+}$ mice accelerate or amplify atherosclerosis progression in Alb*Cc1*$^{fl/fl}$ mice, we would conclude that deletion of hematopoietic CEACAM1 participates in the atherogenic effect of CEACAM1 deletion. We would reach the same conclusion if transfer of WT-derived bone marrow reduces atherosclerosis in *Cc1*$^{-/-}$ and Alb*Cc1*$^{fl/fl}$ mice. Conversely, if WT bone marrow reduces atherosclerosis in *Cc1*$^{-/-}$, but not Alb*Cc1*$^{fl/fl}$ mice, we would then conclude that hematopoietic CEACAM1 does not participate in the atherogenic process.

But how would *Ceacam1* deletion in hepatocytes affect the chronic inflammatory state of the arterial wall? Possibly, through increased triglyceride flux to adipocytes, followed by visceral obesity that leads to macrophage recruitment and the generation of a pro-inflammatory state. This indirect consequence of hepatic *Ceacam1* deletion will support our interpretation of hepatic *Ceacam1* deletion acting as the primary factor in atherosclerosis initiation in this model.

Pitfalls and Alternatives: Hyperinsulinemia-caused by specifically **deleting InsR in liver** does not drive atherosclerosis unless LIRKO mice are fed an atherogenic diet to raise their plasma lipid levels (10). Decreased insulin clearance contributes to hyperinsulinemia in these mice. Thus, it is possible that hyperinsulinemia alone, caused by specific deletion of *Ceacam1* in hepatocytes, does not suffice to drive spontaneous atherosclerotic plaque development in Alb*Cc1*$^{fl/fl}$ null mice. Should this be the case, we will feed global *Cc1*$^{-/-}$, conditional Alb*Cc1*$^{fl/fl}$ null mutants, and liver-specific *Cc1*$^{-/-xliver+}$ rescue mice an atherogenic

Western type diet (WTD; 21% milk fat, 0.15% cholesterol) beginning at 1 month of age for 8-52 weeks (31), and test if the liver-specific deletion confers predisposition to atherogenesis, and whether it accelerates the vascular phenotype in parallel to the acceleration of insulin resistance (3-4 vs 6 months of age). Under these conditions, the rescue mouse will serve to test whether liver-specific CEACAM1 gain-of-function protects against diet-induced atherosclerosis even after prolonged WTD.

It remains possible that we will not detect vascular phenotypic differences between global *Cc1*$^{-/-}$, liver gain-of-function (*Cc1*$^{-/-xliver\ +}$) and liver-loss-of function (Alb*Cc1*$^{fl/fl}$) models because of the small plaque size in *Cc1*$^{-/-}$ mice during this early stage of the atherosclerotic development. Should this be the case, we will feed mice WTD, as this is expected to amplify phenotypic difference between the mice, and as such, would provide a better means to address the question of this Aim. In this case, we might observe correlates of advanced lesion formation, such as macrophage apoptosis and necrosis (77).

In the event the results in Alb*Cc1*$^{fl/fl}$ null mice are not conclusive, we will test our hypothesis using the L-SACC1 mouse with hepatocyte-specific inactivation of CEACAM1. This mouse exhibits hyperinsulinemia with hypertriglyceridemia, but without hypercholesterolemia when propagated on a mixed BL6xFVB background (66). We are currently propagating the mutation on BL6 genetic background to examine the vascular phenotype. If hypertriglyceridemia persists on BL6 background and L-SACC1 mice develop atherogenesis, we would attribute this vascular abnormality to the synergistic effect of hyperinsulinemia and hypertriglyceridemia.

If neither loss-of-function nor gain-of-function in liver CEACAM1 demonstrates a role for this protein in atherogenesis, we will hypothesize that endothelial CEACAM1 is protective against the pro-atherogenic effect of hyperinsulinemia. As a mutually non-exclusive hypothesis, we propose to examine the role of CEACAM1 in endothelial cells in Aim 2.

C2. Aim 2: Examine cell-autonomous effects of CEACAM1 in endothelial cells that predispose to endothelial dysfunction and atherosclerosis.

Hypothesis: **Because CEACAM1 is a shared element between insulin and VEGF-induced Akt/eNOS activation, it is likely that altered signaling through CEACAM1-dependent pathways disrupts endothelial cell's response to insulin and VEGF, and in this cell-autonomous fashion, drives profound endothelial dysfunction and initiates atherosclerosis.**

C2.a. Significance/Rationale: The role of vascular endothelial insulin resistance in the pathogenesis of atherosclerosis has been highlighted by findings indicating that ablation of InsR in this cell type promotes atherosclerosis and endothelial dysfunction by reducing eNOS activation in a cell-autonomous manner (11). Consistent with dependence on eNOS activation by the InsR/Akt1 pathway in response to insulin (78, 84), global ablation of Akt1 promotes atherosclerosis by reducing eNOS activity and increasing vascular cell inflammation and apoptosis without causing significant metabolic abnormalities (83, 87). It also increases vascular permeability and alters blood vessel maturation (82). Despite this demonstration of a critical role for insulin resistance in vascular endothelial cells to the pathogenesis of atherosclerosis, **the mechanism of endothelial cell dysfunction caused by endothelial cell insulin resistance is only partly known.**

CEACAM1 exerts multiple actions that could affect atheroma formation: (*i*) it down-regulates the inflammatory response in activated T cells (36); (*ii*) it mediates VEGF-induced angiogenesis in human vascular endothelial cells (88) as well as promoting new vessel formation and activation of collateral arteries in mice (37); and (*iii*) it reduces vascular permeability (38). Thus, CEACAM1 can potentially regulate endothelial function and vascular integrity. In fact, **global *Ceacam1* deletion causes abnormal vascular permeability and endothelial dysfunction leading to atherosclerosis even on a regular diet and without propagating the mutation on either *Ldlr*$^{-/-}$ or *ApoE*$^{-/-}$ background** (preliminary data). This effect could be mediated by reduced Akt1/eNOS activation by VEGF-A (38) and insulin, as demonstrated by blunted effect of insulin on Akt1/eNOS activation in bAEC cells transfected with *Ceacam1* SiRNA (preliminary data). Thus, **CEACAM1, a shared substrate of insulin and VEGFR2 receptors (38, 81), links insulin to VEGF actions with regard to eNOS activation**. From a mechanistic standpoint, it should be noted that eNOS function is regulated, among other things, by its sub-cellular localization near the plasma membrane (89). Thus, it is possible that CEACAM1 anchors the eNOS protein complex near the plasma membrane, providing the necessary structural underpinning for its action. In fact, upon its phosphorylation by the insulin receptor, CEACAM1 binds to fatty acid synthase (FAS) (22) that has been shown to promote eNOS translocation to the plasma membrane for activation (89). Regardless of whether CEACAM1 activates eNOS by this or other mechanisms, its regulation of vascular integrity requires its phosphorylation by VEGFR2 (38). Thus, we

postulate that **deletion of *Ceacam1* in endothelial cells links insulin resistance in endothelial cells to defective VEGF signaling, causing a marked reduction in eNOS activation by both insulin and VEGF.** This will lead to atherosclerotic plaque formation with limited angiogenic response to VEGF (90, 91). Development of early, but not progressive atherosclerotic lesions in the global *Cc1*$^{-/-}$ mouse supports this hypothesis. Thus, we will investigate whether (*i*) conditional deletion of *Ceacam1* gene in endothelial cells is sufficient to cause atherosclerosis, and *(ii)* conversely, whether restoring CEACAM1 exclusively in the endothelial cells of *Cc1*$^{-/-}$ mice prevents this abnormality. Identification of a CEACAM1-dependent shared mechanism of insulin and VEGF signaling in atherosclerotic plaque initiation will promote better therapeutic and preventive modalities.

Table 4:

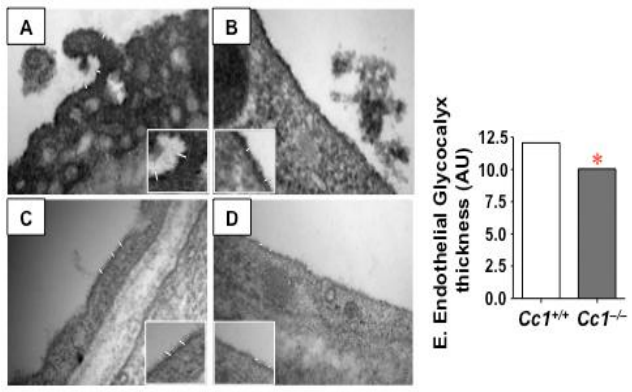
	Cc1$^{+/+}$	*Cc1*$^{-/-}$
Ang-1	0.98 ± 0.24	0.25 ± 0.01 *
Ang-2	0.37 ± 0.05	0.10 ± 0.02 **
VEGF-A	9.53 ± 1.38	3.40 ± 0.41 **
VEGF-C	0.14 ± 0.02	0.10 ± 0.01
VEGF-D	0.62 ± 0.08	0.72 ± 0.10
VEGFR1	1.91 ± 0.22	0.60 ± 0.10 ***
VEGFR2	1.31 ± 0.13	0.50 ± 0.06 ***
VE-Cadherin	0.34 ± 0.04	0.12 ± 0.02 **
β-Catenin	8.22 ± 0.73	4.83 ± 0.36 **

mRNA in aorta of 6 month-old male mice
** p<0.01, ** p<0.001, ***p<0.0001 vs. Cc1$^{+/+}$;(n ≥ 6)*

C2.b. Preliminary data:

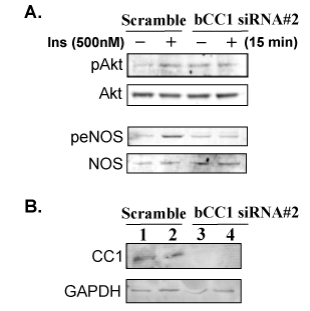
Reduced expression of pro-angiogenic and endothelial cell specific markers in aortic tissue of Cc1$^{-/-}$ mice – mRNA levels of potent pro-angiogenic factors such as the vascular endothelial growth factor A (VEGF-A) and its receptors, VEGFR-1 and -2, as well as angiopoietins (Ang-1 and -2) (79) were significantly **down-regulated** (by ~ >3-fold) in the aorta of *Cc1*$^{-/-}$ mice (Table 4). The decrease in VEGFR-2 mRNA was confirmed by Western analysis that demonstrated a ~40% reduction of aortic VEGFR-2, a major receptor in signalling pathways underlying VEGF-mediated angiogenesis and VEGF-induced vascular permeability. Also significantly reduced were aortic mRNA (not shown) and protein (Fig 3E-F) levels of VE-cadherin, a major transmembrane adhesion molecule of the endothelial adherent junctions that preserves endothelial integrity and permeability (92-94). In contrast, mRNA levels of VEGF-C and VEGF-D, which are mainly involved in lymphangiogenesis, were not altered (Table 4). These data propose a marked decrease in angiogenesis in *Cc1*$^{-/-}$ mice.

Fig. 7:



Reduced endothelial glycocalyx in the aorta and coronary arteries of Cc1$^{-/-}$ mice – Reduced endothelial glycocalyx, a vascular protective network of membrane bound glycoproteins and proteoglycans covering the endothelial cell surface, is associated with endothelial dysfunction, increased leukocyte-endothelial interaction and increased vascular permeability (95, 96). Electron microscopic analyses with digital quantification on fine sections (80 nm thick) obtained from aortic and cardiac tissue revealed a significant reduction of the height of the endothelial glycocalyx in both aorta and coronary arteries of *Cc1*$^{-/-}$ mice in comparison to their WT counterparts (Fig. 7 A-E).

Fig. 8:



Reduced insulin's activation of Akt1/eNOS in bAEC transfected with Ceacam1 SiRNA – bAEC cells were transfected with either scrambled or bovine *Ceacam1*-specific siRNAs [bCC1 siRNA#2 oligo- (38)] to knockdown CEACAM1, as demonstrated by absence of CEACAM1 in the Western blot in Fig. 8B (lane 3-4 vs 1-2). Cells were then incubated in serum-free medium, treated with (+) or without (–) insulin for 15 min prior to analyzing Akt1/eNOS phosphorylation by Western blot (Fig. 8A). Like VEGF (38), insulin induced Akt1 and eNOS phosphorylation (and activation) in cells transfected with scrambled RNA (+ vs – lane). In contrast, insulin failed to activate Akt1/eNOS in bCC1 siRNA *Ceacam1* cells (+ vs – lane).

C.2d. Proposed experiments:

i. Generation of the experimental models:

The endothelial cell-specific VECadCc1$^{fl/fl}$ null mouse – We have followed the same strategy shown in Aim 1 above to generate the conditional endothelial cell-specific null *Ceacam1* mouse. Briefly, we crossed

Cc1$^{loxp/loxp}$ mice with transgenic mice expressing Cre under the transcriptional control of the VE-Cadherin gene promoter (*VECad*Cre) (97) propagated on the BL6 background (Jackson Laboratories). Heterozygous mice have been backcrossed 5 times with BL6 mice. We will backcross at least one more time before breeding to homozygosity. Cell-specific null deletion will be confirmed by Western analysis of CEACAM1 protein levels in MLEC derived from these mice and in endothelial cells derived from aorta, liver, lung, and heart. Special emphasis will be placed on demonstrating aortic deletion of the target gene, as variable levels of recombination have sometimes been seen in this location. A variety of other tissues and cells will also be screened, including peripheral blood mononuclear cells [PBMC; FACS sorted per our routine protocol (98)] in order to rule out any potential leakage of *VECad*Cre, as has been reported for Tie2 (11). We will use male littermates: *VECad*Cre+*Cc1*$^{loxp/loxp}$ [or ***VECadCc1***$^{fl/fl}$]; *VECad*Cre+*Cc1*$^{+/+}$ [or ***VECad*Cre**]; and *VECad*Cre – *Cc1*$^{loxp/loxp}$) [***Cc1***$^{lox/lox}$].

The endothelial cell-specific rescue Cc1$^{-/-xEC+}$ ***mouse –***Using transgenic mice overexpressing CEACAM1 under the control of the endothelial cell–specific *Tie2* promoter (CC1 $^{endo+}$) (37), we will generate Ceacam1 knockouts with an endothelial cell-specific rescue, hereafter named ***Cc1***$^{-/-xEC+}$ and their control littermates: ***Cc1***$^{-/-}$; ***Cc1***$^{+/+}$ and CC1 $^{endo+}$ (**EC-CC1** for simplicity). The strategy employed is the same as in Aim 1. We will examine endothelial cell-specific expression by Western analysis in MLEC, aorta, heart, liver, and lung. Based on our success in this strategy (Aim 1), we are confident that we will achieve the goal in a timely manner. For phenotyping, we will use male littermates at 3-9 months of age.

Generation of *VECadCc1*$^{fl/fl}$ ***on the Ldlr***$^{-/-}$ ***background –*** Although *Ceacam1* mutation is unique in causing spontaneous atheroma in the absence of *ApoE* or *Ldlr* null mutations, the atherosclerosis phenotype of *VECadCc1*$^{fl/fl}$ mice can be milder than that of *Cc1*$^{-/-}$ owing to the absence of hyperinsulinemia (Aim 1). Thus, the difference between its phenotype and that of *Cc1*$^{-/-xEC+}$ rescue mice would be difficult to discern. Thus, we plan to propagate mice onto the BL6.*Ldlr*$^{-/-}$ null background, as described above. We will not use the *ApoE*$^{-/-}$ background because of the close proximity of *ApoE* and *Cc1* loci on chromosome 7.

ii. Phenotyping: We will follow the same metabolic and vascular phenotyping strategy as above. We will evaluate macrophage function/viability, since the *ldlr*$^{-/-}$ background is expected to promote progression to more advanced lesions. To this end, we will examine advanced atherosclerotic lesions, including foam cell formation and apoptotic cells in lesion, by measuring cholesterol ester accumulation following exposure of freshly isolated peritoneal macrophages to oxidized LDL (oxLDL). Because prolonged exposure to this lipoprotein causes apoptosis, we will also measure cell necrosis and apoptosis by FACS analysis after incubation with (oxLDL) (83).

Anticipated Results: Like mice with specific InsR mutation in endothelial cells on the *ApoE*$^{-/-}$ background (11), we do not expect significant alteration in the metabolic phenotype, including cholesterol and lipoproteins profile, of *VECadCc1*$^{fl/fl}$ and *ldlr*$^{-/-}$*VECadCc1*$^{fl/fl}$ mice, especially in light of intact hepatic insulin clearance and action. In contrast, we expect a dramatic adverse effect on endothelial function and integrity, with a more pronounced vascular phenotype in *ldlr*$^{-/-}$*VECadCc1*$^{fl/fl}$ compared to *VECadCc1*$^{fl/fl}$. This will be partly attributed to defective insulin and VEGF signaling via the Akt/eNOS pathway. Thus, *VECadCc1*$^{fl/fl}$ and *ldlr*$^{-/-}$*VECadCc1*$^{fl/fl}$ mice are expected to exhibit a decrease in eNOS activity with an increase in oxidative stress (including ROS generation and a nitroso-redox imbalance). Reduced Akt1 signaling could lead to apoptosis in MLEC, indicating early endothelial dysfunction and atheroma formation. Because Akt1 signaling and Ceacam1 levels in macrophages from *VECadCc1*$^{fl/fl}$ are likely to be intact, we do not expect to observe abnormalities of macrophage viability and function, nor do we expect to see changes in foam cell formation during the progression of atherosclerosis even on the *ldlr*$^{-/-}$ receptor knockout background since this occurs independently of Akt1 (83).

Endothelial integrity and function are key parameters of vascular homeostasis and they depend essentially on orchestrated action of pro-angiogenic factors, particularly VEGF-A and its receptors, VEGFR 1 & 2 (99-101). Consistent with CEACAM1's role in promoting angiogenesis (91) and maintaining physiologic expression levels of angiogenic factors such as VEGF-A and its receptors in endothelial cells (102), *Cc1*$^{-/-}$ mice exhibited a significant reduction of VEGFA and VEGFR-2. Based on induced angiogenesis in CEACAM1^{endo+} transgenic mice with endothelial cell-specific overexpression of CEACAM1 (37), it is likely that reduced angiogenesis in *Cc1*$^{-/-}$ mice is primarily due to CEACAM1 deletion from endothelial cells. In fact, MLEC derived from *Cc1*$^{-/-}$ mice exhibit defective VEGF-mediated NO production, consistent with defective VEGF-dependent *in vivo* permeability in *Cc1*$^{-/-}$ mice (38). A concomitant **decrease in VEGFR and other pro-angiogenic factors, including Ang-1 (103) and VE-cadherin (92),** that are essential for endothelial integrity and vascular stability is predicted to arise in *VECadCc1*$^{fl/fl}$ and *ldlr*$^{-/-}$*VECadCc1*$^{fl/fl}$ mice. Together **with defective VEGFR/eNOS signaling**, this will increase vascular permeability, as measured by the reduction in

their glycocalyx content (preliminary data) and a robust transmigration of inflammatory cells into the sub-endothelial space and initiation of atherosclerosis (94). Thus, we expect that relative to controls and *Cc1*$^{-/-xEC+}$ rescue mice, the number of firmly adhering leukocytes will be higher in all *Ceacam1* deletion mutants (*Cc1*$^{-/-}$, *VECadCc1*$^{fl/fl}$ and *ldlr*$^{-/-}$*VECadCc1*$^{fl/fl}$ mice), as in mice with InsR deletion from the endothelial cells (11). Given that initiation of atherosclerotic lesions is associated with increased endothelial expression of VCAM-1 (48, 49), we expect increased levels of VCAM1 in aorta, lung and MLEC of *VECadCc1*$^{fl/fl}$ and *ldlr*$^{-/-}$ *VECadCc1*$^{fl/fl}$ mice, with a more pronounced phenotype in double mutants. The latter are expected to exhibit a marked decrease in NO with a reciprocal increase in inflammatory signals (51) (52, 53). This will demonstrate that *Ceacam1* deletion from endothelial cells plays a key role in changes in the endothelial barrier and endothelial dysfunction, which contribute significantly to atherosclerosis in *Cc1*$^{-/-}$ mice.

Contribution of CEACAM1 in hematopoietic cells to leukocyte-endothelium interaction will be assessed in **mononuclear cell transfer** from *Cc1*$^{-/-}$, *ldlr*$^{-/-}$*VECadCc1*$^{fl/fl}$, *VECadCc1*$^{fl/fl}$, *Cc1*$^{-/-xEC+}$ and their controls into wild-type recipients. A higher number in firmly adhering leukocytes from *Cc1*$^{-/-}$ relative to *ldlr*$^{-/-}$*VECadCc1*$^{fl/fl}$ and *VECadCc1*$^{fl/fl}$ donors would indicate a role for monocytic CEACAM1 in the vascular phenotype. If the number is the same between *Ceacam1* deletion mutants, *Cc1*$^{-/-}$ and *Cc1*$^{-/-xEC+}$, we would exclude a significant role for monocytic CEACAM1 in atheroma formation, as was shown with regard to monocytic InsR in endothelial InsR mutant mice (11).

To evaluate whether bone-marrow derived neutrophils and macrophages lacking Ceacam1 (as in *Cc1*$^{-/-}$ and *Cc1*$^{-/-xEC+}$) affect plaque development differently from WT cells (*ldlr*$^{-/-}$*VECadCc1*$^{fl/fl}$, *VECadCc1*$^{fl/fl}$ and WT), we will perform bone marrow transplants. If bone marrow-derived cells from global *Cc1*$^{-/-}$ or *Cc1*$^{-/-EC+}$ mice, but not WT, accelerate or amplify atherosclerosis progression in *ldlr*$^{-/-}$*VECadCc1*$^{fl/fl}$ and *VECadCc1*$^{fl/fl}$ mice, we will conclude that deletion of hematopoietic CEACAM1 acts in synergy with the atherogenic effect of endothelial CEACAM1 deletion. We would reach the same conclusion if WT-derived bone marrow reduces atherosclerosis in *Cc1*$^{-/-}$, *ldlr*$^{-/-}$*VECadCc1*$^{fl/fl}$ and *VECadCc1*$^{fl/fl}$ recipients. If on the other hand, WT-derived bone marrow reduces atherosclerosis in *Cc1*$^{-/-}$, but not *ldlr*$^{-/-}$*VECadCc1*$^{fl/fl}$ or *VECadCc1*$^{fl/fl}$, we would conclude that hematopoietic CEACAM1 does not regulate the vascular phenotype brought about by deleting endothelial *Ceacam1*.

Mechanistically, we will investigate whether CEACAM1 anchors eNOS to the membrane by immunohistochemical analysis of CEACAM1 and eNOS in MLEC cells following treatment with insulin and VEGF-A, and by co-immunoprecipitation analysis of CEACAM1/eNOS and FAS in membrane-enriched fractions. We anticipate binding and increased membrane localization in MLEC cells derived from WT, *Cc1*$^{-/-EC+}$ and controls, but not *Cc1*$^{-/-}$, *ldlr*$^{-/-}$*VECadCc1*$^{fl/fl}$ and *VECadCc1*$^{fl/fl}$ mice. To test whether CEACAM1 phosphorylation is required for eNOS recruitment to the membrane, we will examine CEACAM1-eNOS complex formation and co-localization in bAEC cells transfected with CEACAM1 (WT and its site-directed mutants) (38). We expect to observe more binding between phosphorylated CEACAM1 and eNOS, as we have observed for CEACAM1/FAS interaction (22). It should be noted that all reagents are available at the PI's laboratory.

Pitfalls and Alternatives: It is possible that endothelial *Ceacam1* deletion alone does not suffice to drive atherogenesis even on the *ldlr*$^{-/-}$ background. We would then feed mice a WTD, as described earlier with the expectation that this would drive atherogenesis, as was the case for the ApoE.Akt1 double mutant (83).

A regulatory role for CEACAM1 in insulin action in endothelial cell has not been identified. In hepatocytes, CEACAM1 regulates insulin action by promoting its intracellular transport. Whether it exerts a similar mechanism in endothelial cell is unclear. In light of the controversy of the effect of insulin transport on insulin action in endothelial cell, we will make no assumption on the role of CEACAM1 in this process. Thus, it is possible that despite the increased vascular permeability associated with defective CEACAM1 function and decreased signaling to eNOS, endothelial-specific *Ceacam1* mutation does not suffice to initiate atherogenesis. On the other hand, mutating insulin signaling in endothelial cells did not cause atherosclerosis in the absence of a permissive background (11), perhaps because of a compensatory increase in VEGF/eNOS signaling. **This outcome of our experiment would thus clarify whether insulin and VEGF act in a synergistic manner to maintain normal endothelial function and vascular permeability in a CEACAM1-dependent manner.**