Unravelling molecular mechanisms in atherosclerosis using cellular models and omics technologies

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Abstract

Despite the discovery and prevalent clinical use of potent lipid-lowering therapies, including statins and PCSK9 inhibitors, cardiovascular diseases (CVD) caused by atherosclerosis remain a large unmet clinical need, accounting for frequent deaths worldwide. The pathogenesis of atherosclerosis is a complex process underlying the presence of modifiable and non-modifiable risk factors affecting several cell types including endothelial cells (ECs), monocytes/macrophages, smooth muscle cells (SMCs) and T cells. Heterogeneous composition of the plaque and its morphology could lead to rupture or erosion causing thrombosis, even a sudden death. To decipher this complexity, various cell model systems have been developed. With recent advances in systems biology approaches and single or multi-omics methods researchers can elucidate specific cell types, molecules and signalling pathways contributing to certain stages of disease progression. Compared with animals, *in vitro* models are economical, easily adjusted for high-throughput work, offering mechanistic insights. Hereby, we review the latest work performed employing the cellular models of atherosclerosis to generate a variety of omics data. We summarize their outputs and the impact they had in the field. Challenges in the translatability of the omics data obtained from the cell models will be discussed along with future perspectives.

Keywords: atherosclerosis; two dimensional (2D) models; Shear stress and circumferential stretch models; three-dimensional (3D) models; omics technologies

1. Introduction

Atherosclerosis is a progressive and complex vascular disease that, primarily in combination with thrombosis, causes cardiovascular ischaemic events (1). It is associated with a gradual ageing process and recognized risk factors including dyslipidaemia, diabetes mellitus, hypertension, obesity, sedentary lifestyle, cigarette smoking, family history, and psychosocial factors (1). Atherosclerosis develops in the vessel wall of large- and medium-size arteries and involves the build-up of fibrous and fatty deposits, called plaques, ultimately leading to a complex plaque that impedes blood flow. Acute clinical manifestations such as myocardial infarction or stroke are the result of rupture or ulceration of a “vulnerable” atherosclerotic plaque (2). Besides their lipid content, atherosclerotic lesions are characterized by different cell types including inflammatory cells (e.g. macrophages, dendritic cells and lymphocytes), vascular smooth muscle cells (SMCs), and necrotic cell debris underneath a monolayer of endothelial cells (ECs) that lines the interior vessel wall (3). Moreover, some impact of dysfunctional adipocytes in the perivascular adipose tissue has been proposed (4).

During the initial stage of the disease, prolonged exposure to cardiovascular risk factors can lead to endothelial dysfunction, and increased vascular permeability, enabling lipid entry and their modification within the vessel sub-intima. These lipids then act as pro-inflammatory mediators stimulating expression of adhesion molecules and chemotactic proteins by EC, which in turn initiates blood monocyte recruitment. In the intima, recruited monocytes differentiate into macrophages and adopt modified lipids to become foam cells. Simultaneously, the medial SMCs are transformed from a

contractile to a proliferative state and migrate into the intima where they synthesize extracellular matrix (ECM), thus generating the fibrous cap. Progressive structural remodelling of the atheromatous lesions leads to apoptosis of vascular SMC and foam cells, suppresses efferocytosis to give rise to secondary necrosis, inflammation and protease release, which in turn degrades the fibrous cap and increases susceptibility of plaque rupture (5). Both adaptive and innate immune responses drive the chronic inflammation and play crucial roles in determining plaque vulnerability. High-risk plaques are more functions of their composition than of their size. Typically, these "vulnerable" plaques harbour a large acellular lipid-rich necrotic core with an overlying thin fibrous cap infiltrated by inflammatory cells, thrombi and calcium deposits (6). Recent *in vivo* imaging studies in humans showed that superficial erosion is an increasing cause of arterial thrombosis (7).

1.1 A brief overview of cellular systems for the study of atherosclerosis

Different preclinical models have been developed during the past decades for the study of the pathophysiology of atherosclerosis and for the assessment of the efficacy of new therapies. These models can be divided into two major types: a) *in vivo* models (predominantly rodents); and b) *in vitro* (or *ex vivo*) cell models. The *in vivo* models and their advantages/disadvantages will be reviewed elsewhere. *In vitro* models of atherosclerosis, although incapable of mimicking the complexity of this multifactorial disease, can provide opportunities to study mechanisms and generate

large amounts of data such as single or multi-omics data in a more efficient and economical manner. Compared with animal models, *in vitro* models are cost-effective, easily adaptable for high-throughput experiments, offering mechanistic insights within a short timeframe (8). The present review attempts to provide an overview and an update of the field of *in vitro* modelling of atherosclerosis. We review latest work employing single cell (2D) models), either primary cells or cell lines of various tissue origins or from induced pluripotent cells (iPS) shear stress/circumferential stretch models including orbital shaking platforms and parallel plate or stretch chambers, and three dimensional (3D) models including hydrogel-based models, spheroids, bioengineered arteries, arteries on a chip, tissue-engineered blood vessels and 3D vascular networks (**Figure 1**). Some of the above models have been used extensively in the past to delineate the molecular mechanisms of atherosclerosis, while others are still under development and have not gained widespread use but exhibit promising potential for future research. Furthermore, the cellular models of atherosclerosis have been used to generate a variety of omics data (bulk or single cell omics) and we summarize here their outputs and the impact they are having in the field (**Figure 2**). Challenges in the integration and the translatability of the omics data obtained from the cell models will be discussed at the end along with future perspectives.

1.2 Two-dimensional (2D) cell models for atherosclerosis

1.2.1 Endothelial cells

Under normal conditions, ECs control vascular tone, fibrinolytic processes, the traffic of plasma molecules and inflammatory cells and angiogenesis (9, 10). After an acute exposure to atherosclerotic risk factors, ECs are transiently activated and start to express adhesion molecules, cytokines, and chemokines, which mediate leukocyte recruitment into the arterial wall (11-13). Prolonged stimulation of ECs causes their dysfunction: ECs acquire a pro-oxidant, pro-inflammatory, vasoconstrictor and pro-thrombotic phenotype, paralleled by an increased permeability of the endothelial barrier (14-17). Considerable heterogeneity of ECs along different vascular beds has been described and was confirmed during inflammation by single cell RNA-sequencing (18, 19).

To evaluate the functions of ECs in atherosclerosis, numerous studies have been carried using 2D-cell models. Primary cultures of ECs isolated from large vessels such as human umbilical vain ECs (HUVECs), human aortic ECs (HAECs), human coronary artery ECs (HCAECs) and pulmonary artery ECs (PAECs) exposed to different risk factors were studied **(Table 1)** (20-24). However, primary EC models are relatively difficult to use because they maintain the original phenotype for a limited time, have a low lifespan and can generate donor-dependent results (18). In this context, immortalized EC lines have been developed including the EA.hy926 cell line, obtained by fusion of human HUVECs with human lung carcinoma cells. Differences between EA.hy926 cells and primary HUVECs have been reported, such as lesser sensitivity to

pro-diabetic stimuli and higher stress tolerance compared to primary HUVECs (25). Compared to HAECs, EA.Hy26 cells were less efficient in binding and internalizing high-density lipoproteins (HDLs) probably due to the lower activation of Ras and Rap1 signalling pathways (26). Human microvascular ECs (HMEC-1 line) are obtained by transfecting human dermal microvascular ECs with SV40T sequences and exhibit similar characteristics to HUVECs (18), although a lower sensitivity to TNFα was reported (27). HMEC-1 is a good model for studying HDL functionality because these cells are expressing Scavenger receptor class B type I (SR-BI) and promote HDL internalization (28). Human microvascular ECs and EA.hy926 cells exposed to LPS/TNFα and HUVECs exposed to high glucose helped to identify Ninjurin-1 (Ninj-1) as a new player involved in the atherosclerotic process, (29-31). Using the EA.hy926 cells, it was demonstrated that oscillating glucose compared to constant high glucose resulted in a more pronounced inflammatory stress and increased the expression of transendothelial transport proteins (31). A comparative study on human cortical microvascular cell line, primary human brain endothelial cells and primary HAEC demonstrated that human brain ECs restrict low-density lipoproteins (LDLs) transcytosis and degrade them while the HAECs take up LDLs mostly for transcytosis (32).

Cultured ECs were used to investigate HDL functionality in different pathologies (21, 22, 33). It was shown that HDLs from peripheral artery disease (PAD) patients failed to protect EA.hy926 cells from TNFα inflammatory aggression. This dysfunction was associated with the decrease of antioxidant enzyme paraoxonase 1

(PON1) and increase of the pro-oxidant enzyme myeloperoxidase and the chaperone protein clusterin in HDLs particles, these features being aggravated in diabetic PAD patients (33). Moreover, the ROS production of HAECs exposed to HDLs from diabetic patients was positively correlated with the levels of oxidized fatty acids contained in HDLs (21). The model of TNFα-activated HUVEC was used to provide evidence that HDLs from Familial Combined Hypolipidemia (FHBL2) subjects do not lose their ability to preserve endothelial homeostasis as compared to normal HDLs (22). Surfaceome nanoscale organisation analysis applied to HAECs revealed that HDL binding to SRBI together with other 60 cell surface proteins (including CD13) form a so-called "HDL synapse" that directly influences HDL uptake by ECs (34). TNFα-activated EA.hy926 cells were used to evidence that conditioned medium from CRISPR/dCas9-activated enterocytes that overexpressed endogenous apolipoprotein AI and PON1, reduces the inflammatory and oxidative stress in ECs, by attenuating the expression of TNFR1, p22phox and MCP-1 (35).

HUVEC have been also used to prove that exposure to trimethylamine N-oxide (TMAO) originating from microbiota affects ECs permeability by decreasing ZO-2 and occludin, through NLRP3 inflammasome/Cathepsin B activation (20).

ECs are highly flexible and may be engaged to a mesenchymal transition (EndoMT), a process in which ECs progressively lose their endothelial specific markers while gaining mesenchymal and smooth muscle cell markers. In this process, pro-inflammatory molecules, proliferation and secretolytic rates increase, while cell–cell adhesion proteins are downregulated, enhancing the migration process (23). Studies on

HUVEC, HAEC, and HCAEC showed that many factors and signalling pathways driving this transition are also involved in CVD (23, 24). Using TGF-β-exposed HAECs, it was demonstrated that miR-200a inhibits the expression of endothelial-mesenchymal transitions markers blocking EndoMT (36). Whether EndoMT is protective or not in the context of the atherosclerotic process remains to be established.

1.2.2 Macrophages

Macrophages used in *in vitro* experiments are either primary cells or cell lines. The most widely used primary cells are the peritoneal macrophages extracted from mice. Human macrophages are derived from monocytes from peripheral blood, as well as from umbilical cord blood (37). The monocytes can be differentiated to macrophages using 5-400 mM phorbol 12-myristate 13-acetate (PMA) for 24-72 hours. Macrophage differentiation can also be induced by treatment with 10-100 ng/mL macrophage colony-stimulating factor (MCSF) or GC-MSCF (38, 39).

The most widely used macrophage cell lines are the murine RAW264.7 and J774.2 (40), or those differentiated from human monocyte cell lines THP-1 or U937. Macrophages were used *in vitro* to evaluate their roles in foam cell formation, cholesterol efflux, polarization, inflammatory response, matrix remodelling, apoptosis, pyroptosis and efferocytosis (**Table 1**). In THP-1-derived macrophages exposed to inflammatory stress, unmodified LDL uptake is increased via LDL receptor, representing an alternative pathway for foamy-macrophage formation (41).

Cholesterol efflux capacity is used as a measure of HDL functionality. Many clinical studies regarding the correlation between cholesterol efflux and the incidence

of CVD have recently been reviewed (42). The study of the cholesterol efflux from macrophages and the discovery of the molecules that potentiate this process have a great impact on atherosclerosis treatment, but also for other diseases such as oncologic pathologies (glioblastoma) as recently shown (43).

In the atherosclerotic lesions, macrophages present distinct phenotypes namely M1 (pro-inflammatory) and M2 (anti-inflammatory). M1 macrophages, located mainly in the shoulder region of the atherosclerotic plaque and exposed to proinflammatory stimuli, contribute to plaque destabilization and necrotic core formation whereas M2 macrophages produce high levels of anti-inflammatory cytokines such as interleukin-10 and transforming growth factor-beta (44),(45),(46). *In vitro*, macrophage polarization to M1 can be induced by treatment with 100 ng/mL IFN-γ and 10 ng/mL lipopolysaccharide (LPS) and to M2 by treatment with 15 ng/mL IL-4 (47).

In vitro studies revealed that in response to the inflammatory stress, the inflammatory mediators, including cytokines, chemokines, and reactive oxygen species are released from the macrophages. Moreover, under endotoxin stress, apoE expression in macrophages is decreased through a mechanism that implies NF-κB activation (48) a process partially reverted by metformin (49).

Macrophages contribute to the remodelling of the extracellular matrix, influencing plaque stability. *In vitro* studies showed that MMP expression is upregulated during macrophage differentiation and the extracellular matrix production is influenced by the M2 polarization of macrophages (50).

Macrophages undergo cell death (pyroptosis) as a critical step in the formation of the necrotic core in the atherosclerotic plaque, while the clearance of apoptotic cells by efferocytosis protects against plaque progression and rupture. The mechanism of apoptosis and pyroptosis induced by oxidized LDL, as well as the processes and molecules that protect macrophage from ox-LDL-induced apoptosis was elucidated (51), (52), (53) while the mechanism of efferocytosis was only partially described Efferocytosis may involve mitochondrial calcium uniporter complexes influencing calcium concentration and ROS generation modulate efferocytosis (54) and upregulation of Sirt1 mediated autophagy (55). Studies on peritoneal macrophages from apoE3 and apoE4 knock-in mice reveal impaired efferocytosis, increased apoptosis, and endoplasmic reticulum stress in apoE4-expressing macrophages (56).

1.2.3 Hepatocytes

Metabolic liver diseases (metabolic dysfunction-associated fatty liver disease (MAFLD) and metabolic dysfunction-associated steatohepatitis (MASH)) are associated with atherosclerotic CVD risk (57). Hepatocytes are the most abundant cell types in the liver (>80%), followed by Kupffer cells and other cell types. Hepatocyte-like models are the most frequently used and about 40 human hepatic tumor cell lines are available. The most commonly used are HepaRG, Huh7, SK-Hep-1, Hep3B and HepG2 (58). HepG2 cells exhibit hepatocyte key features and experiments based on them have been reported in over 34,000 papers and over 500 records associated to the keywords “HepG2” and “atherosclerosis” in PubMed, making HepG2 cells the most widely used model (59). They were used to study hepatocyte lipoprotein metabolism

and triglyceride accumulation, including the effect of related drugs **(Table 1)** (60, 61). In addition, HepG2 were used to evaluate the effects of natural and synthetic active compounds, related to cardio-metabolic health, including curcumin (62), bergamot (63), ginsenoside (64), kaempferol (65), extracts from traditional African spices (66) and others. Oleic acid/palmitic acid-treated HepG2 cells were proposed as a cell-based model of hepatocyte fat accumulation and fibrosis (65, 67). Despite some differences between normal hepatocytes and HepG2 cells, the latter are useful to address several issues related to atherosclerosis pathophysiology and its pharmacological management (59).

1.2.4 Adipocytes

The adipose tissue externally associated to the cardiovascular system (perivascular adipose tissue (PVAT) and epicardial adipose tissue (EAT)) displays anti-inflammatory and anticontractile activity, but, when dysfunctional and hypertrophic, may contribute to the atherosclerotic process (68, 69), in line with similar observations regarding excess/dysfunctional visceral adipose tissue (VAT) (70, 71). The availability of adipocyte cell models, especially taking advantage of co-culture systems with other cells types involved in the atherosclerotic process, allows to unveil specific aspects of their interaction with atherosclerotic plaque cells. Specifically, the secretome from dysfunctional adipocytes shows an altered adipokine pattern, with reduced protective molecules (*e.g.*, adiponectin) and increased proinflammatory ones (e.g., leptin, resistin), which may promote macrophage migration/differentiation and VSMC phenotype change and migration toward the intima via paracrine mechanisms (72).

Mouse 3T3-L1 pre-adipocytes, the most widely used murine model, were developed from murine Swiss 3T3 cells and exhibit a fibroblastic phenotype that can be induced to differentiate to pre-adipocytes, with lipid droplet accumulation (73). The PPAR-γ agonist rosiglitazone also stimulates differentiation in a dose-dependent manner (74). Interestingly, induction of expression of the chemokine CXCL14 in 3T3-L1 cells resulted in M2 polarization of RAW264.7 macrophages, recapitulating a similar event in PVAT cells in ApoE-/- mouse, also resulting in M2 polarization of plaque macrophages (75). The 3T3-F442A cell line, also from Swiss 3T3 cells, allows to obtain larger adipocytes, with a greater accumulation capacity.

Mouse OP9 cells are stromal cells derived from newborn mice genetically deficient in functional macrophage colony-stimulating factor (MCSF). OP9 cells differentiate to adipocytes after reaching confluence and maintain the differentiation achieved for long periods in culture (76). In OP9 cells, C/EBP-α, C/EBP-β and PPAR-γ are involved in the differentiation process, similarly to normal human adipocytes (77).

Human adipocyte models are less commonly utilized although very promising. Adipose-derived stem cells are isolated from samples of human fat, resulting from surgery (78) and can be differentiated to adipocytes. Their main disadvantage is their limited capacity for renewal and the low amount of cells collected from adipose specimens.

Human SW 872 liposarcoma cells originate from a malignant undifferentiated liposarcoma tumour. SW 872 cells are pre-adipocyte-like cells, with an immature adipocyte phenotype and may be differentiated to mature adipocytes by oleic acid

treatment showing similarities with adipocytes and lipid drops accumulation. Some important adipocyte genes (PPAR-γ, PPAR-α, lipoprotein lipase (LPL), CD36, cholesterol ester transfer protein (CETP)) are constitutively expressed in SW 872 cells (79-81). Upon oleic acid treatment, SW 872 cells become apparently dysfunctional adipocytes and show reduced uncoupling protein-1 (UCP-1) gene expression, increased pro-inflammatory cytokine release, reduced glucose uptake and increased oxidative stress (81), which is also specifically present in PVAT from obese subjects (82). Interestingly, supernatant of activated SW 872 adipocytes has been shown to promote migration of THP-1 monocytes (83). The modulation of these pathways by natural compounds with protective activity has also been tested in SW 872 cells (81) and seems promising also in the context of the PVAT-atherosclerotic plaque relationship **(Table 1)**. Further studies are required to fully exploit such pathophysiological dynamics.

1.2.5 Vascular Smooth Muscle Cells

Studies of atherosclerotic plaque composition from human autopsies and animal models have concluded that vascular SMCs contribute to plaque development at all stages by acquiring different cellular phenotypes. Vascular SMC phenotype switching occurs both in the intima and the media, characterized by less-differentiated SMCs including myofibroblast-like cells, macrophage-like cells, mesenchymal stem-cell-like cells and osteochondrogenic cells. Single-cell models of SMCs have been often applied to assess the effect of atherogenic stimuli on SMCs phenotype change. The trans-differentiation to a macrophage-like state can be observed by cultured aortic mouse

SMCs loaded with free cholesterol complexed to methyl-β-cyclodextrin, which are water-soluble, cyclic polysaccharides that can enhance the solubility of hydrophobic compounds (84). Similarly, SMCs cultured with aggregated or oxidized low-density lipoprotein have downregulated elastogenic capacity and increased macrophage foam cell markers. Additionally, these transformations were accompanied by the acquisition of macrophage-like function as assessed by phagocytotic activity. Recently, a meta-analysis comparing the average expression profiles among several *in vitro* and *in vivo* datasets demonstrated that cholesterol treatment of cultured SMCs failed to recapitulate the full extent of cell state transitions observed in murine models of atherosclerosis (85). Compelling *in vitro* evidence showed that the treatment of cultured SMCs with platelet-derived growth factor-BB (PDGF-BB), oxidized lipoproteins and phospholipids, inflammatory cytokines, and lysophosphatidic acid, induces a phenotypic switching from contractile SMCs to fibroblast-like cells (86-90). This conversion is characterized by loss of contractile markers with gain of synthetic organelles, migratory and proliferative properties involved in fibrous tissue formation for the plaque cap and neointimal hyperplasia (91). However, we still miss corroborative direct evidence for a role of these factors in directly controlling SMC phenotype *in vivo*. The phenotypic transition of SMCs to an osteochondrogenic-like state plays a major role in orchestrating vascular calcification and stiffness. The best *in vitro* models mimicking *in vivo* vascular calcification is to culture SMCs in calcifying medium characterized by high phosphate (3.8 mM) and calcium (2.4 mM) concentration, 15% fetal calf serum and neutral phosphate donor (NaH_2PO_4 / Na_2HPO_4)

(92, 93). At day 7 of culture, the expression of osteoblastic genes runt-related transcription factor 2 (RUNX2), receptor activator of nuclear factor kappa-B ligand (RANKL), osteopontin (OPN), osteoprotegerin (OPG) and alkaline phosphatase (ALP) genes was increased **(Table 1)**. Finally, although the experimental implementation of a single-cell in vitro model is easily arranged and cost-effective, it cannot report on the interaction between different cell types and the continuous exposure of vascular SMCs to mechanical stimuli thereby encouraging more advanced in vitro models.

1.2.6 Endothelial Progenitor Cells

Endothelial colony forming cells (ECFCs) – also known as outgrowth endothelial cells (OEC) or late endothelial progenitor cells (late EPC) - constitute a key endogenous system that contributes to vascular integrity and homeostasis. ECFCs function is achieved by promoting angiogenesis and maintaining vascular homeostasis through the secretion of matrix proteins, growth factors and cytokines (94). ECFCs resemble ECs phenotypically, genetically, and functionally. In addition, genetic and epigenetic changes associated with ECs often exist in ECFCs, making them excellent surrogates for ECs (95). ECFCs can be procured in a minimally invasive manner and their use provides an attracting vascular disease model to probe mechanisms of endothelial pathogenesis and delineate therapeutic targets in atherosclerosis (96). At present, *in vitro* studies on functional capacity of ECFCs are limited.

Alexandru et al. (97) obtained late EPC from mononuclear cells (MNCs) isolated from peripheral blood of hypertensive–hyperlipidaemic (HH) hamsters, an experimental model of diet-induced atherosclerosis and healthy hamsters as controls.

Importantly, hamster late EPC morphology and characteristics were found to be similar to those from humans (98). In addition, late EPCs from the animal model of atherosclerosis (late EPC-HH) exhibited different characteristics and functions than late EPC of healthy origins (late EPC-C). Specifically, late EPCs-HH were smaller and irregular in shape and presented a decreased expression of endothelial surface markers (97) which suggested that EPC immunophenotyping may be very powerful in predicting endothelial dysfunction in atherosclerosis. Functional analyses such as proliferation, adhesion, migration, angiogenesis, vasculogenesis and inwardly rectifying potassium (Kir) channels activity showed the functional differences between late EPC-HH and late EPC-C confirming EPC dysfunctionality in atherosclerosis. The altered EPC function in atherosclerosis was attributed to the low expression of miRs and defective activation of insulin-like growth factor 1 (IGF-1)(97).

The recovery of late EPC functionality affected by atherosclerotic diet administered to hamsters, was assigned to the effects of microparticles (MPs) or microvesicles (MVs) of healthy origins and their ability to transfer these miRs to late EPCs and improve their function as a result of IGF-1 activation (97). These data provided an important and novel perspective for decoding the complex mechanism of MV role in the cell–cell communication and EPCs-mediated vascular repair. Moreover, they suggested that the inhibition or overexpression of specific miRNAs in MVs delivered into recipient cells (such as late EPC) may represent a promising therapeutic tool to treat the vascular diseases (97).

Similarly, it has been shown that *in vitro* exposure of atherosclerotic hamster late EPCs to platelets of healthy origins increases their functional properties (99). These findings highlighted a new biological role for platelets in regulating EPC function in CVD, thus helping to develop new therapies based on targeting the interplay between platelets and EPCs.

The *in vitro* data from murine late EPC are consistent with those from humans. Thus, a study on late EPC obtained from patients with chronic obstructive pulmonary disease and established atherosclerosis, revealed that the number of EPC clusters, their migration, vasculogenesis, as well as the expression of CXCR4, were significantly decreased, meaning that these cells were dysfunctional and their dysfunction was closely related to vascular endothelium alteration (100).

Similar investigations were carried out on *ex vivo* cultures of ECFC obtained from human peripheral blood MNC. Thus, a recent study showed that ECFCs from patients with atherosclerotic CVD present a decreased colony forming capacity and impaired angiogenic properties, in a manner dependent on the severity of the disease (96). Reduced proliferation, migration, tube-like structure formation and NO production were also described in ECFC from patients with CAD (101-103). Furthermore, cultured ECFC from peripheral blood MNC of patients with atherosclerotic CAD were used to examine the mechanisms involved in the individual CAD susceptibility, particularly the role of dysregulated redox signalling (104). It was observed that, compared to ECFC from healthy controls, ECFC from CAD patients were less proliferative as evidenced by lower Ki67 expression and prolonged

417 population doubling time, and showed deficiency in migration as seen by delayed
418 wound healing capacity *in vitro* and significant decline in angiogenesis potential (105).
419 The same study highlighted a significant association between ECFC
420 tetrahydrobiopterin (BH4) levels, their wound healing capacity and angiogenesis
421 potential. Moreover, oxidative stress induced in ECFC *in vitro* with 3-morpholino
422 sydnonimine (SIN-1) it was correlated with reduction in BH4 levels and diminished
423 migration, proliferation, and angiogenesis (105).

424 Sheng et al. reported that the outgrowth endothelial cells (OECs) isolated from
425 MNCs of patients with CAD displayed a significant decrease in the percentage of
426 $CD34^{+}/CD45^{-}$ population, in colony formation, proliferation and tubulogenesis, as well
427 as in NO production, endothelial nitric oxide synthase (eNOS) activity, and the
428 phosphorylation level at Ser1177 of eNOS, compared with late OECs isolated from
429 control participants (106). In addition, endothelium-dependent flow-mediated
430 vasodilation was positively correlated to OEC functions including NO production,
431 eNOS phosphorylation, colony formation, and proliferation (106).

432 The deterioration of functionality of late EPCs, measured by their tube
433 formation capability in *in vitro* Matrigel assays, was also shown in patients with
434 ischemic stroke (107). Recently, it was revealed that the regenerative functions of late
435 EPC can be enhanced by EPCs-derived extracellular vesicles obtained from human
436 umbilical cord blood, when used at a dose higher than the physiological dose, without
437 altering their endothelial identity (108).

Interesting and somewhat in contradiction with the studies presented previously, it was shown that blood outgrowth EPC isolated from CAD patients display mature and fully differentiated EC characteristics (103). These types of EPCs were positive for EC markers CD31 and CD105 and negative for CD133 and hematopoietic markers CD45 and CD14. Furthermore, another study showed that in a direct co-culture model late outgrowth of CAD EPCs exhibited confluence over SMCs for 7 days and maintained a confluent layer, attached to SMCs depending on the α5β1 integrin and displayed alignment with flow (109). These data validated peripheral blood EPCs isolated from CAD patients as suitable candidates for the endothelialisation of the small-diameter tissue-engineered blood vessels (TEBVs). Many *in vitro* studies have revealed the dysfunction of late EPCs in CVD but studies on the restoration of the function of these cells by MVs, extracellular vesicles or exosomes, apart from those presented above, have not been identified in the literature **(Table 1)**.

1.2.7 Induced pluripotent stem cells (iPSC)

The ground-breaking progress achieved so far with cellular reprogramming has increased the interest in the use of induced pluripotent stem cells (iPS cells) as a conceivable source for generating various types of vascular cells for large-scale experiments. Being patient-derived, iPS cells retain the disease-causing mutation, thus could generate isogenic vascular structures that are perfectly relevant samples for diverse pathological contexts (110).

Induced PS cells are derived from reprogramming adult cells into pluripotency, typically by using four transcription factors, Oct4, Sox2, Klf4, and c-Myc (111). In this

state, cells can be committed and differentiated into almost any cell type, including mesoderm-derived lineages for modelling atherosclerosis. A high level of complexity can be achieved with multicellular co-cultures, which allows generation of 3D tissue-like constructs mimicking *in vivo* physiology.

The principles of generating vascular cells from iPS cells relies on the capacity of iPS cells to give rise to different germ layers, among which is the mesoderm. Mesodermal cells can be then further manipulated towards hematopoietic differentiation (to generate neutrophils, monocytes, macrophages, mast cells or lymphocytes), endothelial differentiation or smooth muscle cell specification. Successful differentiation can be certified by expression of specific markers.

1.2.7.1 iPSC-derived Endothelial cells

The most commonly used methods to differentiate ECs rely on embryoid body assembly of iPS cells in the presence of growth factors (BMP4, Activin A, bFGF, VEGF) that promote mesoderm induction and endothelial lineage specifications, followed by cell purification and further expansion in conditions favouring EC growth (112-114). The efficiency varies depending on the type and dose of growth factors (115). Alternative methods also exist (116), which are based on seeding iPS cells on a matrix-coated plate a stromal cell feeder layer, as well as treatments with various molecules in a timed fashion that dictates the progressive iPS cell differentiation through an intermediate mesoderm differentiation phase toward the EC lineage (112, 116, 117). Recently, a higher efficiency in generating ECs has been reported, based on

the delivery of modified mRNA encoding the transcription factor ETV2 at the intermediate mesodermal stage of differentiation (118).

Importantly, all these protocols suffer from limitations, most notably in the low yield and high degree of heterogeneity, as they generate a mixture of ECs of all three major subtypes (arterial, venous and lymphatic) (119-121). Induced PS cell-derived ECs were reported to exhibit standard response to inflammatory signals and production of NO and to enhance angiogenesis, tissue perfusion and organ function (116, 122). Achieving a particular phenotype of mature ECs by *in vitro* differentiation of iPS cells was proved difficult and a refined method of arterial-venous differentiation of ECs has not yet been reported. Specific tissue microenvironments and blood flow hemodynamic were used in order to impact cell differentiation (123). By varying the concentration of VEGF-A in the induction medium, the selective enrichment in arterial or venous lineages was reported, while the presence of VEGF-C appeared to favour iPS cell differentiation into lymphatic phenotype (124). The use of 3D scaffolds during the differentiation process proved to increase the efficacy but still the percentage remained low (125). Specific subtypes of ECs were obtained by selecting an adequate source of cells for re-programming into pluripotency and by optimizing *in vitro* differentiation conditions to generate a physiological system.

1.2.7.2 iPSC-derived SMC

SMC are commonly derived from iPS cells *in vitro* through exposure of mesodermal precursors to PDGF-BB (126-128). The reported approaches yielded highly pure SMC subtypes without the need for enrichment through cell sorting.

A protocol of human iPS cell conversion into SMC reported in 2015 by Patsch lab may serve as a standard for deriving both ECs and vascular SMCs at scales relevant for drug discovery and regenerative therapies (114). Recently, SMCs were reportedly obtained from iPS cells through a stepwise differentiation protocol, in which the intermediate lineages were generated in precisely defined media composed of certain combinations of growth factors and small molecules (127). Although several vascular SMC subtypes were obtained with this protocol, the timely-controlled treatment with a MEK inhibitor was shown to induce a more mature and contractile phenotype. The fate decision in vascular smooth muscle phenotypes (synthetic or contractile) during the differentiation process could be also instructed by the time spent in culture and the presence of serum and growth factors. (128).

1.2.7.3 iPSC-derived Hematopoietic Cells

Guided differentiation of iPS cells into various types of hematopoietic cells was also reported. Neutrophils can be produced from iPS cells with ETV2 modified mRNA to direct haemato-endothelial commitment, followed by treatment with GM-CSF, FGF-2, and UM171 to expand myelomonocytic progenitors, and G-CSF and retinoic acid agonist Am580 to induce neutrophil maturation (129). Neutrophils generated by this protocol were reportedly closer to foetal than adult neutrophils, in terms of NET production in response to PMA and chemotactic response to IL-8 but still they phagocytosed bacteria and produced ROS efficiently. When overexpression of ETV2 was induced concomitantly with GATA2, multipotent $CD34^{+}CD45^{+}$ hematopoietic progenitors were obtained from iPS cells (130).

525 Macrophages can be obtained through a step-wise differentiation of iPSCs into
526 mesoderm and hematopoietic progenitors, followed by myeloid specifications and
527 macrophage terminal differentiation (131, 132). T lymphocytes can be generated from
528 iPSCs *in vitro* by a three-step differentiation process (133). Unless the CAR technology
529 is used in combination with iPSC technology, the yielded lymphoid cells are
530 unpredictable in terms of antigen specificity (134). Selection of peripheral blood
531 lymphocytes as initial iPSC sources may also result in relevant T cell populations, as
532 iPSCs generated from T cells with defined TCR specificity retain the configuration of
533 the parental cell and the rearranged TCR loci remain unchanged during in vitro
534 differentiation (135).

535 ### 1.2.8 Co-culture models

536 A variety of co-culture models involving vessel wall resident cells (ECs, mural
537 cells, and fibroblasts) and immune/inflammatory cells (i.e. monocytes, macrophages)
538 have been employed to better mimic the *in vivo* processes and provide valuable insights
539 into the intricate dynamics of cell-cell communication. Cellular models involving two
540 or more cell types have shown transcriptional profiles like those observed from tissue
541 studies due to more physiological paracrine associations and feedback loops, metabolite
542 exchange, diffusion/transportation of a variety of mediators (soluble factors,
543 extracellular vesicles, regulatory ncRNAs) (136).

544 Both direct and indirect two-dimensional (2D) cellular systems allow culturing of
545 mixed populations to form a monolayer or aiding cell compartmentalisation using
546 devices, like trans-well inserts used for cell paracrine communication studies (137).

However, this setup requires prior knowledge on molecule gradients and permits studying only unidirectional responses (from donor to recipient cells) (8).

Due to an increased complexity in establishing a stable co-culture system, most available models focus on two cell types at the time. However, models with 3 different cell types have been developed, facilitating the rapid screening of numerous drugs, allowing for detailed monitoring of how drugs impact the interactions between cells (138). A study from Noonan and colleagues established a simple platform to study the interaction between the vascular (VSMC and ECs) and immune compartment (THP1 macrophages), showing that the established multidirectional communication better portrays *in vivo* cellular responses compared to all the co-culture combinations based on 2 cell types (139) **(Table 1)**. In recent years some experimental models have evolved to account for biomechanical alterations. Work from Liu et al, have shown that multicellular platforms can be exploited to study atherosclerosis at nascent and intermediate disease stages via different cell-matrix and cell-cell combination strategies (140). In particular, the authors showed that the addition of shear stress to the triple cell culture (HCAC, HCASMC and human THP-1) promoted increased oxidized low-density lipoprotein (oxLDL) levels and a proinflammatory status as observed *in vivo.*

1.3 Shear stress and circumferential stretch models

The vascular endothelium is affected by biomechanical forces induced by blood flow. The velocity and direction of blood flow vary at a spatiotemporal level depending on both the phase of the cardiac cycle and the vascular geometry, thereby regulating

endothelial cell function and gene expression (141, 142). The arterial endothelium is not only subjected to a frictional force called wall shear stress (WSS), but the pulsatile changes in blood pressure generate simultaneously a cyclic circumferential stretch (CCS) on the cells. Together, these biomechanical forces regulates functional, structural, metabolic, transcriptomic and epigenomic changes in ECs through mechanosensory and mechanosensitive signalling pathways, thereby determining endothelial homeostasis and the location of arterial diseases, such as atherosclerosis (143). Commonly used *in vitro* models (described below) often aim to study in detail the influence of isolated biomechanical forces on EC behaviour.

1.3.1 Orbital shaking platform

The orbital shaker is a mechanical platform on which two-dimensional EC monolayers in culture plates spin horizontally, subjecting them to repeated cycles of multidirectional shear stress caused by the induced movement of the fluid in the well (144, 145). The shear stress amplitude is determined by the volume, density and viscosity of the culture medium, as well as the radius of the well containing the cells and the radius of rotation (146). This platform enables working with small medium volumes, which greatly decreases expenses if the effects of treatment are studied. The platform's motion creates a multidirectional low shear stress (often called "disturbed flow") in the centre of the well while exposing the edge to a cyclic high unidirectional shear stress (also called "atheroprotective flow") (147, 148). These distinct shear stresses cause a monolayer of ECs to respond differently depending on where they are located in the same well (147, 149), making this model rather comparable to the *in vivo*

situation where regions exposed to atheroprotective and atheroprone shear stress are located adjacently. While this may be seen as an advantage, it may also represent a limitation of this model because ECs in a particular location release factors that influence ECs in the adjacent region. Using orbital shaking platforms, it has been confirmed that disturbed flow generated altered endothelial transcriptome and epigenomic profiles, reprogramming them towards an atherogenic phenotype (150, 151) **(Table 1)**.

1.3.2 Parallel plate flow chambers

EC monolayers can be subjected to a distinct type of WSS, either unidirectional or oscillatory, of defined magnitude using a parallel flow chamber channel. In a closed circulation or in a unidirectional system, a flow of cell culture medium is induced by one pump (or two pumps in series) over ECs cultured on a glass slide, which is coated with polystyrene, gelatine or fibronectin (152-157). In general, the Newtonian equation for incompressible fluids is used to calculate the shear stress applied to an EC monolayer in the parallel flow chamber (158), although it has been shown that the shear stress profile across the chamber area can be non-uniform (159). Parallel plate flow chambers are widely used nowadays and small devices are also commercially available (160, 161). Limitations of these flow chambers include the relatively large amount of cell culture medium required for the experiments, making it difficult to study the effects of hard-to-get or expensive compounds, as well as the risk of contaminations, particularly for long-term experiments (162) **(Table 1)**.

1.3.3 Stretch chamber

ECs undergo a permanent cyclic stretch in the arterial wall. This mechanical force can be mimicked using a custom-built or commercial stretch chamber. Depending on the type of device used, stretch can be bi-axial or multi-axial and varied as an isolated parameter or in combination with WSS, which may exert different effects on EC function (163-167). Although stretching devices have been frequently used for smooth muscle cells, omics studies toward the effects of cyclic circumferential stretch on cultured ECs are scarce (168, 169) **(Table 1)**.

1.4 Three-dimensional (3D) models

In vitro 2D models for atherosclerosis lack the physiological 3D structure present *in vivo*, pose a concern with substrate topography and stiffness, and thus, do not display a pathological milieu resembling the atherosclerotic plaque. In contrast, 3D models have emerged as helpful approaches, which can generate cell constructs that recapitulate the 3D extracellular matrix of the tissue, thus providing both EMC-cell and cell-cell interaction. An optimal 3D *in vitro* model should replicate the complex tissue architecture, including natural ECM, cellular components, and tissue morphology, therefore supporting the emergence of tissue-specific functions. As such, this system is deemed capable of faithfully modelling the pathological characteristics observed in diseased tissues. Significant steps have been taken for the development of *in vitro* 3D models that replicate, at least partially, the complex structures of human tissues, including vascular tissue (170). These innovative models serve as valuable tools for

studying pathological processes and gaining deeper insights into the mechanisms underlying the development of diseases, including atherosclerotic organoids (171).

Another added value of 3D models relies on considering cell-to-cell communication between different cell types, mediated through paracrine mediators, which is one of the main weaknesses of 2D models. The main 3D cell models used for the study of atherosclerosis are described below.

1.4.1 Hydrogel-based 3D models, bioengineered arteries, artery-on-a-chip

The 3D cell-laden hydrogel constructs offer a compelling platform for studying atherosclerosis. They provide a beneficial 3D *in vitro* environment comprising extracellular matrices and the ability to incorporate multiple cell types and factors known to contribute to disease development. One of the first 3D models based on hydrogel developed to study atherosclerosis was a static long-term co-culture setup on fibrin gels (172). Using this model, the authors demonstrated for the first time the feasibility to investigate the accumulation of LDL (172) and immune cells (173) *in vitro* in a sub-endothelial matrix.

A step forward was achieved in 2013 in a study that engineered a 3D artery model based on biodegradable tubular scaffold matrices, mimicking the structural and functional characteristics of a native artery, developed under dynamic pulsatile flow conditions. This native-analogous bioengineered artery model was used to study initial events in atherosclerosis, namely the accumulation of LDL and HDL in the intima as

well as the binding and transmigration of monocytes under dynamic pulsatile flow conditions (172). However, the introduction of a synthetic material-based tubular scaffold effectively prevented the formation of a true-to-life morphology of the arterial wall, presenting a chaotic mixture of myofibroblasts and synthetic material, therefore significantly compromising the modelling power of this approach. With the rise of 3D printing technology, bio-compatible inks and bio-printing technologies have been explored to develop blood vessel models. A 3D printed, cell-laden alginate-di-aldehyde gelatine hydrogel vessel, crosslinked with barium chloride, showed fibroblasts attachment in tubular shaped construct. Despite a very good printability and non-toxic nature, alginate-based hydrogel is well-known for its relatively poor mammalian cell attachment and encapsulation, which greatly limits its application in modelling complex physio-pathological processes (174). By contrast, bio-printing technologies are well-suited to generate vascular phantoms for haemodynamic studies. A gelatine-patterned, endothelialised carotid artery model developed to study the endothelium response to wall shear stress provided insight into the physiological changes that occur in the endothelium of the carotid artery *in vivo* (175). The authors used this model to investigate the variations in endothelial morphology, function and permeability which are associated with the wall shear stress patterns of carotid artery geometry and concluded that it can be effective for studying the pathogenesis of atherosclerosis by which flow dynamics control the endothelium layer function *in vitro* (175) **(Table 1).**

In a typical artery-on-a-chip model, a porous polyester membrane (e.g. polyethylene terephthalate membrane) is inserted into a microfluidic chip to form the

upper and the lower compartments. Endothelial cells and vascular smooth muscle cells are seeded onto the two sides of the polyester membrane to recapitulate the arterial wall. The vascular chip is then perfused by a programmable flow pattern. This model provides an easy and versatile tool to investigate the human vascular cell behaviour in a tissue context, such as oscillatory flow-induced endothelial-to-mesenchymal transition (176). Alternatively, microchannels with narrowing are designed to investigate atherosclerotic plaque-induced haemodynamic alternation and platelet activation (177-179).

In a state-of-the-art approach to specifically investigate the role of EVs and intercellular communication among vascular cell types, the 3D microfluidic systems can be used. These have been only recently employed for such purposes, either by co-culturing two different cell types in such systems (180), or investigating the role of EVs in a blood vessel on-a-chip (181). Despite the great potential of these 3D microfluidic systems to elucidate intercellular communication within the vascular bed, these have not been used so far in the contest of atherosclerosis.

1.4.2 Spheroids

Spheroids are three-dimensional cellular aggregates that provide an experimental model resembling the original tissue. They enable the study of the interaction between one or multiple cell types involved in an atherosclerosis process within the same structure, as well as their interactions with the extracellular matrix. Various methods have been utilized to generate spheroid cultures, including the hanging drop method, centrifugation, spinner flasks, and non-adherent substrates (8).

In a murine model of hindlimb ischemia, hybrid spheroids composed of mesenchymal stem cells and colony-forming endothelial cells demonstrated greater angiogenic potential than spheroids with a single cell type or even adherent cultured cells. This improvement of the angiogenic capacity could enhance the therapeutic efficacy of transplanted cells by promoting the formation of new blood vessels in the peripheral arterial disease (182). Additionally, the critical role of matrix metalloproteinase MMP14 has been identified in the progression of atherosclerotic lesions through a spheroid model of VSMCs in which MMP14 regulates their proliferation, a phenomenon not observed under conventional 2D culture conditions (183).

Through the hanging drop method, spheroids mimicking an advanced-stage atherosclerotic lesion, or a pseudo-plaque have been developed. This bioengineered constriction allows for the study of atherosclerosis aetiology and includes blood-derived myeloid cells as cellular components found in human carotid artery plaques (171). Other spheroid models have also been constructed to assess efferocytosis, the process of apoptotic cell clearance by macrophages, which is impaired in atherosclerosis (184). In this model, by regulating the proportions of macrophages and VSMCs and the concentration of ox-LDL, atherosclerotic spheroids at three different stages were generated, allowing for the evaluation of differences in the expression of genes related to lipid metabolism and inflammation (185).

By studying the change in morphology of VSMC spheroids in response to FAK, Rac, Rho, and Cdc42 inhibitors, a machine learning algorithm was developed to

719 characterize the morphological changes that occur in neointimal formation in vascular
720 diseases (186) **(Table 1)**.

721 ### 1.4.3 Tissue-engineered blood vessels

722 The research of (TEBV) is primarily driven by the clinical shortage of
723 autologous vascular graft for vascular reconstruction surgeries. Human cell-based
724 tissue engineered blood vessels (TEBVs) hold great potential to develop into novel *in*
725 *vitro* human model of atherosclerosis, or human-to-animal xenograft model of
726 atherosclerosis. In pre-clinical and clinical applications, a natural artery serves as the
727 best template for development of TEBVs. The attempts to fabricate artery-like TEBVs,
728 however, have been hampered by methodological difficulties in the past decades.

729 L'Heureux and colleagues pioneered the development of the first artery-like
730 TEBV by rolling confluent smooth muscle cell (SMC) and fibroblast sheets onto a rod
731 (the 'cell-sheet method') to form a TEBV (187, 188). The complex fabrication and
732 maturation procedure required 7-9 months to complete and the reproducibility was poor
733 with a success rate <20% (189, 190). The spiral, rather than concentric, structure of the
734 vascular layers posed a serious risk of intraluminal delamination, leading to life-
735 threatening thrombosis and aneurysm (191, 192). Nevertheless, the cell-sheet method
736 is the first attempt to construct a triple-layer TEBV comprising the endothelium,
737 medium and adventitia, and still inspires today's research in this field. A recent
738 development of this method introduced an external polymer sheath to accelerate TEBV
739 maturation (190). Notably, despite this exciting progress, there is no follow-up study or

similar report published in the past five years, which again, raises the question of the reproducibility of the cell-sheeted based approaches.

Alternatively, seeding cells into a synthetic porous scaffold (the 'scaffold-seeding method') became a popular way to fabricate hybrid TEBVs without reproducibility problems (193, 194). When subjected to perfusion culture, collagen synthesis can be induced in the TEBV to achieve a bursting pressure of ~2000 mmHg, comparable to human saphenous vein (193). However, the porous scaffold disrupts the formation of continuous vascular cell layers leading to a severe morphological defect. The degradation products of synthetic material, in the case of bio-degradable scaffold, stimulates chronic inflammation, SMC de-differentiation and endothelial cell (EC) detachment (195, 196), making it unsuitable to model human artery. To date, fabrication of an artery using cultured human cells is no longer a fantasy. However, major development is still required to tackle the obstacle of poor reproducibility before meaningful translational applications can be developed, including modelling human atherosclerosis.

1.4.4 3D vascular networks

Recent advances allow vessel modelling *in vitro* and *in vivo* through derivation of renewable vascular cells and promising data engineering 3D vascular networks have already been reported (122, 197). Thus, one study used both iPS cell - derived ECs and iPS cell-derived SMCs seeded onto nano-fibular scaffolds to generate vascular grafts (198). Alternatively, by aggregating iPS cells in 3D suspension in the presence of growth factors and small molecules, human vascular organoids have been produced that

showed successful integration into immunodeficient mice and further maturation post-transplant, thus giving rise to vascular networks containing arteries, veins and capillaries (199). Induced PS cell -derived vascular cells can also be mixed with various biomaterials (ECM proteins and growth factors) and cultured on specifically designed scaffolds to promote tissue growth (125, 200). By culturing iPS cell-derived SMCs in mixture with ECM gel (collagen or fibrin), a dense cell sheet has been obtained, which was then shaped to a tubular structure as tunica media. The inner lumen of this tubular structure was next filled with iPSC-derived EC suspension and further incubated to allow uniform attachment of ECs on the wall (200). All these strategies offer the advantage of being able to produce patient-specific vasculatures for therapeutic purposes.

Importantly, proper remodelling and maturation of *in vitro*-produced vascular networks are important prerequisites to become fully functional and integrated into the surrounding tissue. Moreover, it is essential to acknowledge the limitations of any *in vitro* model and consider how the results can be validated *in vivo*, in human disease contexts.

2 Overview of omics studies using human or mouse cells in atherosclerosis

Atherogenesis, the process of atherosclerotic plaque formation, is a multifaceted phenomenon involving inflammation, tissue remodelling, and cellular trans-

differentiation. In this comprehensive review we delve into the intricate pathophysiology of atherogenesis reporting recent single or multiomics studies and summarize key findings, and insights presented in research papers between 2019-2024.

2.1 Summary of transcriptomic studies

Instead of a single contractile or synthetic state, SMCs exhibit a spectrum of phenotypes, whereby they transition to an intermediate multipotent cell state termed "SEM" cells (expressing the stem cell, endothelial, and monocyte/macrophage markers Ly6a, Vcam1, and Ly6c1 respectively) (201). SEM could then differentiate into macrophage-like and fibrochondrocyte-like cells, capable to revert to SMC phenotype. Transcriptome analysis of modulated SMCs from mouse and human atherosclerotic arteries revealed upregulated fibroblast-related genes including Tcf21 (Transcription factor 21, a causal coronary artery disease gene) that is required for phenotypic modulation (202). Interestingly, increased TCF21 expression was associated with a reduced risk of coronary artery disease in humans (203).

Over 80% of SMCs in advanced mouse atherosclerotic lesions lose expression of contractile markers and exhibit phenotypes of other cell linages. Up to 30% of SMCs gain expression of Lgals3/Mac2, whereas smaller percentage express stem cells or myofibroblasts markers, like Sca1/Ly6a, or the platelet-derived growth factor (PDGF)-β receptor (204). Krüppel-like factor 4 (*Klf4*) directly modulates the phenotypic changes of SMCs in atherosclerotic lesion, targeting the promoter of SMC marker genes in phenotypically modulated SMCs (90). Studies in cultured SMCs treated with

cholesterol identified > 800 KLF4 target genes including many that regulate pro-inflammatory responses (204). ScRNA-seq and scATACseq on human carotid atherosclerotic plaques demonstrated a dominant synthetic phenotype of SMCs (expressing COL1A1, MGP and COL3A1), limited number of which was *KLF4*$^{+}$ indicative of conversion from SMCs to either a synthetic or macrophage-like phenotype (205).

Slenders *et al* developed an integrative single-cell transcriptomics-driven workflow rooted in human large-scale genetic studies, identifying putative candidate genes and affected cells associated with cardiovascular traits (206). The authors identified candidate genes associated with CAD, coronary artery calcification, and plaque enriched in ACTA2^{+} SMCs cell population. Amongst SMC-enriched candidate genes selected for functional testing, *Kank2*, *SkI*, and *Ednra* correlated with the level of calcification, proliferation, and migration in the cells of ascending aorta, making them interesting targets for further functional studies.

The diversity of ECs across various vascular beds attribute to their heterogeneity and plasticity. ECs exposed to lysophospholipids can differentiate into innate immune cells. By RNA-seq analysis of HAECs incubated with lysophosphatidylcholine (LPC) or lysophosphatidylinositol (LPI) it was shown that both compounds activate molecular mechanisms that transdifferentiate HAECs into innate immune cells, by inducing the danger-associated molecular pattern receptors, expressing major histocompatibility complex class II molecules and T-cell co-stimulation / co-inhibition receptors (207). Using the same model, IL-35 and IL-10

826 could reverse mitochondrial ROS-mediated acute and innate immune trans-
827 differentiation responses, but could spare metabolic reprogramming and trained
828 immunity signatures, which are not dependent on mitochondrial ROS (208). Most
829 recently, TMAO was shown to promote reprogramming of HAECs at transcriptomic,
830 kinomic, and metabolic level, thus inducing transdifferentiation into innate immune
831 cells through upregulating cytokines/chemokines, secretomes, and clusters of
832 differentiation (CDs). Based on RNA-Seq, scRNA-Seq, and kinome analyses, it has
833 been revealed that TMAO upregulated 190 genes in HAECs, activating the
834 phosphorylation of 12 kinases, together with PKR-like ER kinase (PERK) pathways,
835 and inducing the innate immune memory functions (trained immunity) (209).

836 High Density Lipoproteins (HDL) are promising therapeutic targets and tools
837 for atherosclerosis due to multifunctional properties like efflux of cholesterol from
838 macrophage foam cells, anti-oxidative, anti-inflammatory, anti-microbial, anti-
839 apoptotic and endothelial integrity, amongst others (210, 211). However, the
840 mechanisms by which rHDL exerts its various atheroprotective and anti-inflammatory
841 functions are still not understood. Using transcriptomics, Theofilatos et al. (212)
842 investigated the endothelial signalling pathways and the genes that could contribute to
843 atheroprotection by rHDL containing human apoA-I. For this purpose, HAECs were
844 treated with rHDL-apoA-I and their total RNA was analysed with whole genome
845 microarrays. It was found that 410 transcripts were significantly changed in the
846 presence of rHDL-apoA-I.

In another study, Valanti et al. (213) investigated the atheroprotective potential of rHDL particles containing human apolipoprotein E3 (apoE3) using transcriptomics in HAECs treated with rHDL-apoE3. It was found that rHDL-apoE3 treatment caused changes in the expression of 198 genes mainly involved in re-endothelialization and atherosclerosis-associated functions.

2.1.1 Summary of omics studies in endothelial cells using shear stress and circumferential stretch models

With the large availability of defined *in vitro* shear stress models, several laboratories have advanced this field by combining it with omics approaches to profile the endothelial transcriptome (both mRNAs and non-coding RNAs), DNA methylome and proteome in an unbiased manner, which has helped to identify novel mechanosensitive signalling pathways in arteries. These numerous studies have been summarized in several excellent reviews (143, 214-218). In brief, these omics approaches have unveiled that shear stress regulates transcriptional programs involved in critical cellular processes such as apoptosis, proliferation, migration, inflammation, oxidative stress, unfolded protein responses and endothelial-to-mesenchymal transition (EndMT). In addition, shear stress epigenetically controls the expression of genes that regulate DNA methylation such as H3K27ac with downstream effects on Hippo-YAP-TAZ, Notch and WNT signalling. Proteomic approaches uncovered shear stress-regulated proteins involved in vascular development and remodelling such as VEGF, ANG2 and TGFβ family members. Altogether, these unbiased studies have importantly

contributed to further our understanding of the initiation and focal distribution of atherosclerosis. However, like with all omics approaches, the future challenges lay in the integration of data from different experimental shear stress models, variability in origin of the ECs used and the chosen experimental conditions.

2.2 Summary of sc-transcriptomics studies

Applying single-cell omics techniques uniquely allows to explore cellular changes within plaques, helping to decode their roles in human subjects or specialized mouse models of atherosclerosis. Respectively, the single cell transcriptomics and single-nucleus chromatin accessibility profiling have shed light on cell type-specific patterns of gene expression and cis-regulatory elements contributing to the development of atherosclerotic lesions. In one study (219), Ord et al investigated chromatin accessibility in cell types within human atherosclerotic lesions, presenting a resource of 7000 snATAC-seq profiles covering 5 major cell types: ECs, macrophages, B cells, T/natural killer cells, and SMCs. The findings aligned with recent scRNA-Seq studies, revealing cell subtypes, and providing high-resolution maps of regulatory elements. They confirmed master regulators like PU.1 and CEBPA for macrophages, TEAD3 and MEF2C for SMCs, while also identifying new subtype-specific TFs. The study linked TF motifs to SMC phenotypic shifts and explored *in vitro* vs. *in vivo* chromatin accessibility, connect to the study by H. Pan, et al., (201).

By leveraging chromatin co-accessibility data, the researchers inferred pairs of chromatin accessibility peaks likely to be in close physical proximity with the 3-dimensional space, potentially encompassing a larger set of target genes than previously anticipated. The research delved into the genetic contributions to CAD and MI. Over 90% of CAD GWAS variants are found in non-coding regions of the genome, making it challenging to identify causal variants (220). Specifically, authors identified cell type-specific snATAC-seq peaks in half of the CAD/MI risk loci, implying cell type-selective mechanisms of action. Authors found substantial overlap, supporting primary cell cultures' use, whereas risk loci for CAD were enriched in enhancers, implying cell-specific mechanisms. Surprisingly, *in vitro* enhancer profiles captured most cell type-specific chromatin accessibility regions, indicating their usefulness as surrogates for *in vivo* epigenetic studies. The study identified risk variants for CAD/MI within open chromatin regions and inferred *cis*-regulatory networks affected by these variants. However, a few developmental TF motifs were enriched specifically in *in vivo*-unique regulatory elements, possibly due to tissue origins, absence of blood flow, or reprogramming of epigenetic and transcriptional states *in vitro.* Further experimental validation is needed to understand functional effects. The study advances understanding of genetic complexity in CAD, offering insights into enhancer-promoter interactions and highlighting novel candidate gene targets. The study limitations were acknowledged, such as the small number of replicates and the use of samples from diseased individuals, still it serves as a valuable resource for studying atherosclerosis-associated cell types.

In a comprehensive study, Turner et al, (221) provide a single-nucleus atlas of human coronary artery chromatin accessibility, covering healthy and atherosclerotic samples from over 40 patients. They provide valuable cell-type-specific epigenomic profiles shedding the light on gene regulation and mechanisms associated with CAD risk, whilst enhancing our understanding of cell plasticity and heritable disease risk in the coronary vessel wall. This atlas uncovers over 323,000 unique cell-type-specific regulatory elements (CREs) in coronary artery cells, with 54% specific to individual or limited cell types, particularly SMCs. The results offer insights into SMC phenotypic modulation, providing information on accessible regions, genes, and potential transcription factor motifs involved in the transition of native SMCs to modulated SMCs, such as fibromyocytes. Integrative statistical genetics and machine learning prioritizes the cell-specific regulatory variants and mechanisms underlying CAD risk loci, linking genetic variants to target gene promoters associated with CAD risk, thus revealing PRDM16 and TBX2 as potential key regulators. However, limitations include incomplete annotation of intermediate cell types, the higher difficulty in nuclei extraction from diseased samples, and an imbalance between the subclinical and advanced lesion samples, due to the lack of available lineage-tracing snATAC-seq datasets. Moreover, the modest sample size for QTL-based studies might have hindered the discovery of numerous context-specific regulatory mechanisms. Functional follow-up studies are suggested to investigate target binding sites and affected SMC processes, shedding light on additional mechanisms of disease risk. Despite a significant step forward in uncovering causal disease processes related to atherosclerosis, study is an

untapped resource for future preclinical studies targeting CAD treatment. Further advancements in single-nucleus and spatial sequencing technologies are expected to improve the discovery of regulatory variants and mechanisms, enabling more comprehensive investigations of coronary artery diseases.

2.3 Summary of sc-proteomics studies

When considering the application of single-cell methodologies for characterizing the cellular proteome, the field lags significantly behind the progress made in single-cell transcriptomics. This is primarily attributed to the challenge of amplifying proteins in a manner comparable to reverse transcription's ability to amplify nucleotides. While the exponential improvement in the sensitivity of advanced mass spectrometers is gradually addressing this limitation, there remains a trade-off between the number of cells analysed and the depth of the analysis (number of proteins quantified). The most recent methodologies detects 1,000 – 2,000 proteins across a few hundred cells (222, 223). In contrast, recently developed methodologies like mass cytometry (MC) and full spectrum flow cytometry (FSFC) utilize flow cytometry to analyse thousands of cells, with a theoretical capacity of up to 100 or 50 proteins, respectively. To date, the use of MC by means of CyTOF has been preferentially selected to address the proteomic landscape of atherosclerosis at a single-cell level. These single-cell atlases have focused primarily on immune cells and show that certain cell subsets are overrepresented within plaques. Using an integrated single-cell multi-omics approach, Winkels et al. showed an increase in leukocyte heterogeneity in mouse

953 atherosclerotic aortas defining 11 principal clusters which appeared to be relatively
954 dominated by T-cell and myeloid cells (224). These clusters displayed a high
955 correlation in their transcriptomic and protein marker signatures, the latter composed
956 of 35 surface markers. A similar approach has been applied by Fernandez et al. to
957 compare carotid artery plaques from patients with symptomatic *vs*. asymptomatic
958 disease. As above T-cells were overrepresented, together with macrophages as myeloid
959 cell representatives (225). Furthermore, plaques from symptomatic patients presented
960 a distinct subset of more activated and differentiated CD4+ T cells depicting T cell
961 exhaustion.

962 ApoE$^{-/-}$ LysM-cre Cd40$^{flox/flox}$ (CD40mac$^{-/-}$) mice, with ablated CD40 signalling
963 resulted in more stable plaques when compared to control ApoE knockout, with an
964 overexpression of a subset of resident-like CD206^{+}CD209b^{-} macrophages (possibly M2
965 anti-inflammatory macrophages) observed (226). These results align with those of Ge
966 *et al.*, who observed an overrepresentation of M1 pro-inflammatory macrophages
967 (CD86^{+}CD68^{+}) in vulnerable plaques from human patients (227). Taking a different
968 approach Abe et al. utilized CyTOF to elucidate the role of the ERK5-NRF2 axis in
969 atherosclerosis. Their findings demonstrated that this axis reprograms myeloid cells,
970 particularly macrophages, inducing a distinct senescence-associated secretory/stemness
971 phenotype, which enables cells to evade cell cycle arrest and promote atherogenesis
972 (228).

973 One of the main challenges of multi-omic approaches remains in the integration
974 of results. Most recently, CITE-seq emerged as a novel technology capable of analysing

975 comparable numbers of proteins to mass cytometry, alongside the RNA content of a
976 single cell, thereby directly providing integrated surface markers and RNA-seq data.
977 This methodology has recently been applied to analyse the atheroma plaque (229).

978 The path towards achieving high-throughput, cost-effective single-cell
979 proteomics, capable of analysing thousands of proteins across significant numbers of
980 cells, remains complex. It is not certain whether or when conventional mass cytometry
981 and single-cell mass spectrometry will reach this milestone. However, recent
982 developments in nanopore technology, applied to protein sequencing are promising
983 (230, 231). This innovative approach has the potential to provide single-cell proteomic
984 data comparable to single-cell transcriptomics (232).

985 2.4 Summary of proteomics studies in human coronary artery
986 endothelial and smooth muscle cells

987 The advances in proteomics have unveiled many complexities regarding use of
988 cellular (and other) models to examine the processes involved in atherosclerosis.
989 The first map of vascular smooth muscle cell proteins was reported on 2001 for human
990 saphenous vein medial smooth muscle cells (233), with this reporting only modest
991 numbers of identifications. Since then, the numbers of proteins identified has increased
992 rapidly, with 235 reported by Mayr et al in 2005 for mouse aortic smooth muscle cells
993 (using 2D-PAGE and MS analysis) (234). Later, gel-free and label-free approaches
994 increased these numbers to 815 (235) and with advances in technology the totals are
995 now >8600 proteins for human coronary artery smooth muscle cells using a data-

independent analysis (DIA) approach (Xu, S.(236)). Lower numbers (3668 proteins) were detected consistently across 6 replicate samples from a single donor using data-dependent analysis with parallel acquisition – serial fragmentation (DDA-PASEF) (237) than with the corresponding DIA-PASEF approach (> 7800 proteins across 3 different donors (236)), indicating the superiority (at least in terms of total identifications) of the DIA method. Similar rapid increases have been made in the detected protein numbers from endothelial cells, with recent studies having detected > 7000 proteins across 3 different donors using DIA-PASEF (Xu, S.; unpublished data). A recent multi-omics study has compared HCAECs with those from the internal thoracic artery (HITAECs) (238). 3325 proteins were detected in total, with 244 differentially upregulated in the HCAECs, and 287 in the HITAECs when compared to the other cell type. These data indicate a degree of heterogeneity at the molecular level between these two subtypes, with the HCAECs showing significant elevations in basement membrane and collagen synthesis and assembly, and formation of intercellular junctions, whereas the HITECs showed augmented pro-inflammatory signalling, protein, and nitrogen compound synthesis, as well as ribosome biogenesis. These proteome data were compared with corresponding transcriptomic analyses.

These numbers represent the total number of unique proteins detected across multiple cell donors, with the numbers detected for a single donor (~7800) being less than those detected across 3 donors in total (236). This indicates significant differences between the proteomes obtained from different cell donors, and highlights the challenges of using cells from single donors, which may not provide representative

data. Data obtained from multiple donors is advantageous, but this may result in a loss of critical data if only proteins present in all samples are considered. These differences may arise from smooth muscle cell plasticity and different degrees of de-differentiation. However, potential contributions from other lifestyle, genetic and disease factors to this variability cannot be eliminated and may be very important. Differences have also been observed, though to a lesser extent, across multiple human coronary artery endothelial cells from different donors. Interestingly, comparison between the available proteomes for smooth muscle and endothelial cells showed a high degree of overlap (e.g. ~5800 common proteins, with ~2800 further proteins unique to smooth muscle and ~1200 unique to endothelial cells (236)) **(Table 2).**

2.5 Summary of metabolomics studies in hepatocytes and adipocytes

HepG2 cells are commonly used as a model for studying liver cancer, drug metabolism and metabolic aspects related to cardiometabolic diseases, due to their ability to maintain most liver-specific functions (59). Establishing HepG2 cell core metabolome is crucial to gain insight into the vital molecular processes of these cells and for practical purposes, such as drug discovery, toxicology research, and disease treatment. Also for HepG2, a combination of spectrometric techniques was used to obtain broad coverage of the metabolic space (239), including nuclear magnetic resonance (NMR) or mass spectrometry (MS) in tandem with gas (GC) or liquid (LC) chromatography. A recent meta-analysis investigated the meta-metabolome of HepG2 cells from 56 projects performed using LC-MS, GC-MS and NMR (240). This

immortalized hepatocyte cell line is one of the most studied closed biosystems from a metabolomic point of view and is in the top three regarding the number of metabolomic publications in PubMed, along with HEK293 and MCF-7 cells. Despite possessing similar genetic backgrounds, different examples of HepG2 cells (genetically modified or treated with various drugs and agents, like for example oleic acid to induce lipid accumulation) can show largely different metabolic phenotypes. This variability could provide insights into the specific metabolism and machinery of these hepatocytes. Information was collected on 15,161 metabolites previously detected in HepG2 cells. On average, it was found that one single metabolomic experiment reported about 331 findings (with a maximum of 13,926 unique metabolites, a minimum of 7 metabolites, and a median of 46 metabolites (240). It has been observed that even in panoramic studies, scientists focus on specific metabolites, ignoring the rest of the metabolomic profile. Interestingly, 288 metabolites are repeated from study to study. This may indirectly confirm their key role in the metabolism of hepatocytes, but also indicate significant limitations of technologies that only allow high-reliability identification for these compounds. The comprehensive list of identified metabolites is often not fully disclosed (241). In addition to artificially narrowing the width of panoramic data, the phenomenon of data closure was also reported, with a landscape of metabolomics data that remains quite ambiguous, in contrast to proteomics (242). Despite advancements in generating high-resolution spectral profiles, interpretation of metabolomic data still largely relies on expert intuition and remains a significant challenge and there is still uncertainty regarding the assessment of retention indices in the published data.

Adipocytes function as both energy storage in the form of lipids, as well as a proper endocrine organ releasing several active molecules, including adipokines. Thus, a metabolomic study of adipocytes may include lipidomic analysis of adipocyte content (243) and a metabolomic assessment of adipocyte secretome (244). Interestingly, primary white, beige, and brown adipocytes show distinct lipidomes reflecting their different organelle composition and cell functions, with major differences between the thermogenic fat cells and the non-thermogenic white adipocytes, since thermogenic adipocytes possess higher contents of phosphatidylethanolamine and phosphatidylcholine fractions (243). Upon adipogenic differentiation, key regulators of adipogenesis are stimulated and cells begin to accumulate lipids. A lipidomics analysis has thus been carried out in undifferentiated and differentiated 3T3-L1 cells, revealing significant changes in lipid content during adipogenesis, including enrichment in intracellular triacylglycerol, increased intracellular free fatty acids, which can be used for subsequent synthesis of other lipids, such as sphingomyelin and ceramide. Enriched intracellular diacylglycerol may provide materials for the synthesis of phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine, lysophophatidylcholine and lysophosphatidylethanolamine (245). No metabolomic data are available so far for human SW 872 liposarcoma cells.

3 Translatability and integration of the omics studies: challenges and opportunities

The development of new state of the art omics technologies has enabled understanding of biological processes, regulatory mechanisms and pathological conditions in a depth that was unthinkable few decades ago. Advancements in single cell omics, particularly, have enabled the clarification of the functions of individual cell populations within a tissue or organ, as well as in diseases pathogenesis. This is particularly crucial in atherosclerosis, given the diverse array of cell types involved in plaque formation, progression and rupture. The omics technologies offer numerous advantages, when applied to cell models, notably in identifying novel biomarkers and gene targets that are crucial for enhancing CVD risk management. Prediction of novel, in combination with existing risk factors, such as LDL cholesterol, diabetes and hypertension alongside genetic factors, demands omics data integration through AI/ML pipelines, posing significant implementation challenges. Integrating single omics data from different cell types and systems (single cells, co-cultures, 3D models) poses further challenges due to variability in experimental setups, sample preparation, ‘omics instrumentation/platforms, and AI/ML workflows. Integration of multi-omics data is even more challenging because certain omics data are more consistent than others. Additionally, the complexity increases when considering data sourced from cells extracted from diverse species, such as, human, mouse, rabbit etc, where significant variations exist in RNA copy numbers and conservation, especially in small RNAs.

Systems biology and multi-omics data integration are cutting-edge approaches in biomedical research. By combining information from various "omics" layers, such as genomics, transcriptomics, proteomics, metabolomics, and epigenomics research will continue to provide comprehensive understanding to advance the atherosclerotic research. Furthermore, combining multi-omics data from patient samples, using resources like PlaqueView database will enable the identification of molecular signatures associated with disease subtypes, progression, and treatment responses (246). This, in turn, could facilitate the development of more effective diagnostic tools, prognostic indicators, and personalised treatment strategies for individuals. As a network, COST Action AtheroNet will aim to facilitate exactly those efforts.

4 Is there a single *in vitro* model suitable to study atherosclerosis?

Great effort from the scientific community has advanced the development of *in vitro* models (247) By embracing this comprehensive approach, we gain valuable insights into cell-cell communication dynamics in atherosclerosis, enhancing the reflection of human vascular complexity and advancing our understanding of drug effects and therapeutic implications. Plate-based co-cultures allow investigating into disease aspects such as endothelial dysfunction, VSMC phenotypic switching and immune cell activation. However, most co-culture studies involve only two cell types, due to increased complexity in establishing a stable system when more cell types are

involved, emphasizing the importance of experimental design and appropriate measurable outcomes to prevent data misinterpretation, especially when incorporating additional cell types and/or factors like flow alterations and drugs. Moreover, engineered vessels are still in their infancy and there are several technical challenges to overcome before obtaining comprehensive experimental models of atherosclerosis 'on-a-chip'. Thus, we are just scratching the surface as available high throughput data is sparse, thus additional studies and omics data cross-validation may permit gaining further insight.

5 The role of COST Action CA 21153 (AtheroNET)

The present review paper discusses the advantages and disadvantages of *in vitro* models currently used for the study of atherosclerosis. Review is supported by the COST Action CA21153 "Network for implementing multiomic approaches in atherosclerotic cardiovascular disease prevention and research (AtheroNET)" and is the joint effort of its members. AtheroNET is aimed at providing a comprehensive framework for researchers interested in cellular and molecular research in the field of atherosclerosis as well as for clinical researchers in this area, empowering translational links between them. The network is focusing on the use of multiple omics technologies and data integration through machine learning/artificial intelligence ML/AI approached to bridge the novel paradigms in prevention, diagnosis, and treatment of atherosclerotic cardiovascular disease (ASCVD). The AtheroNET COST Action (https://atheronet.eu/)

promotes specific initiatives such as the creation of inventories for *in vitro* and *in vivo* experimental models of atherosclerosis, in order to facilitate knowledge transfer; generation of technical notes, relating to the use of the models reported in the above-mentioned inventories; and review articles addressing the specific topics arising from this Action (**Figure 2**).

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used Grammerly to correct English spelling. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Figures

Figure 1: Cell models used for atherosclerosis research with applications. Summary of all different 2-D and 3-D cell models systems used to study atherosclerosis is given and we list potential applications in atherosclerotic research for mechanistic discovery or pharmacological evaluation. Image was created in BioRender.com

Figure 2: Unravelling the mechanisms of atherosclerosis using omics data obtained from cell model systems.

Multiomic approaches using diverse cell model systems have greatly contributed to data generation and latest understanding of molecular processes. We list advantages and limitations to the current state of the art approaches for multiomic data integration when using various *in vitro* cellular models. AtheroNET COST Action CA21153 incorporates multiomic approaches for advancing the field of atherosclerosis and we summarise core deliverables from the Action objectives. As a multinational and cross institutional, pan-European network, we aim to facilitate knowledge transfer and disseminate latest developments. Image was created with BioRender.com

Tables

Table 1: Summary of cell model systems used for the study of atherosclerosis.

Type of model	Cell type	Species	Experimental system for the study of atherosclerosis	Ref.
2D models	**Endothelial cells**			
	HUVEC	human	Model to study the effect of TMAO on EC permeability	(20)
	HAEC	human	Model to study human brain ECs in LDL transcytosis	(32)
	HUVEC, HAEC, HMEC-1, EA.hy926	human	Models to study HDL functionality	(21, 22, 28, 33, 34)
	HUVEC	human	Model to study the role of glucose in transendothelial transport	(30)
	HUVEC, HAEC, and HCAEC	human	Models to study endothelial to mesenchymal transdifferentiation	(23, 24, 36)
	Monocytes/macrophages			
	THP-1	human	Model to study LDL uptake and foam cell formation during inflammation	(41)
	J774	mouse	Model to study cholesterol efflux capacity of apoA-I or HDL	(40)

	PBMCs	human	Model to study macrophage polarization (M1 vs M2)	(47)
	RAW 264.7	mouse	Model to study inflammatory mediators and effect of drugs	(48, 49)
	THP-1	human	Model to study Ox-LDL induced apoptosis	(51-53)
	Primary macrophages	mouse	Model to study efferocytosis	(56)
	Hepatocytes			
	HepG2	human	Lipoprotein metabolism and triglyceride accumulation, response to hypolipidemic drugs	(60, 61)
	HepG2	human	Evaluation of the effects of natural and synthetic active compounds, related to cardio-metabolic health,	(62-66)
	HepG2	human	Model of fatty liver disease	(65, 67)
	Adipocytes			
	3T3-L1	mouse	Model of adipocyte effects on M2 macrophage polarization	(73-77)
	Adipose-derived stem cells	human	Model of adipocyte differentiation	(78)

	SW 872	human	Model of adipocyte differentiation towards dysfunctional phenotype, promoting monocyte migration and effects of natural compounds	(79-81, 83)
	Vascular Smooth Muscle Cells (VSMC)			
	Primary aortic SMC	mouse	Model to study SMC trans-differentiation to macrophages	(84)
	Primary SMC	mouse	Model to study phenotypic switching from contractile SMCs to fibroblast-like cells	(86-90)
	Primary SMC	human, rat	Model to mimic in vivo vascular calcification	(92, 93)
	Endothelial Progenitor Cells (EPC)			
	Late EPC	hamsters	Model to predict endothelial dysfunction in atherosclerosis based on immunophenotyping	(97)
	Late EPC	hamsters	Model to study the role of macrovesicles in cell-cell communication and its therapeutic potential for atherosclerosis	(97)
	Late EPC	hamsters	Model to study the role of platelets in regulating EPC function in CVD	(99)

	Late EPC	human	Model to study EPC dysfunction I patients with obstructive pulmonary disease and established atherosclerosis	(100)
	ECFC	humans	Model to study ECFC dysfunction in patients with CAD	(101-103)
	OECs	human	Model to characterize changes in OECs in patients with CAD versus healthy subjects	(106)
	Late EPC	human	Model to study the regenerative functions of EPCs and the role of EPCs-derived extracellular vesicles	(108)
	EPC (peripheral blood)	human	Model to study endothelialisation of small-diameter tissue-engineered blood vessels	(109)
	Induced Pluripotent Stem Cells			
	ECs	human	Response to inflammatory signals, angiogenesis, tissue perfusion and organ function	(116, 122)
	SMCs	human	Relevant for drug discovery and regenerative therapies	(114)
	Co-cultures			

	VSMC, ECs and THP-1	human	A 3-cell co-culture system to study the interactions between vascular wall cells and immune cells	(139)
	HCAC, HCASMC and human THP-1	human	A 3-cell co-culture system to study the atherosclerosis progression by oxLDL and inflammation	(140)
	Orbital shaking models			
	ECs	mouse	Used as models to study endothelial cell responses in transcriptome and epigenome to disturbed flow	(150, 151)
	Parallel plate flow chambers			
	ECs	various	Model to study disturbances of flow to endothelial cell functions	(158-162)
	Stretch chambers			
	ECs	various	Model to study ECs permanent cyclic stretch in the arterial wall	(163-167)
	HUVECs	human	Model to perform omics studies toward the effects of cyclic circumferential stretch on cultured ECs	(168, 169)
	Hydrogel-based			

	ECs, VSMC, PMN	human	Human neo-intima model used to study the accumulation of LDL and immune cells in the sub-endothelial matrix	(172, 173)
	ECs, SMCs	human	Human 3D artery model to study the initial events in atherosclerosis	(172)
	ECs	human	Endothelialised carotid artery model to investigate the variations in endothelial morphology, function and permeability associated with the wall shear stress	(175)
	Artery on a chip			
	ECs, VSMCs	human	Used as a model to investigate the human vascular cell behaviour in oscillatory flow-induced endothelial-to-mesenchymal transition	(176)
	Microfluidic chips			
	HUVECs	human	Used as a model to investigate atherosclerotic plaque-induced haemodynamic alternation and platelet activation	(177)

3D models	IPSCs	human	BBB model used to study the role of EVs in intercellular communication among vascular cell types	(180)
	Spheroids			
	MSCs, ECs	mouse	Used as a model of hindlimb ischemia consisting of hybrid spheroids to investigate the angiogenic capacity of ECs	(182)
	VSMCs	mouse	Used a model to study the role of MMP14 in the progression of atherosclerotic lesions	(183)
	HUVMs	human	*In vitro* engineering of a three-dimensional human fibroatheroma model	(171)
	Macrophages, VSMCs	rat	Generation of atherosclerotic spheroids at different stages to evaluate differences in the expression of genes related to lipid metabolism and inflammation	(185)
	Primary VSMCs	human	Used as a model to assess pharmacological efficacy through ML/AI	(186)

	Tissue-engineered blood vessels			
	SMCs, fibroblasts	human	First artery-like TEBV by rolling confluent SMCs and fibroblast sheets onto a rod	(187, 188)
	ECs, VSMCs	human	Scaffold-seeding method of fabrication of hybrid TEBVs	(193, 194)
	3D vascular networks			
	iPSC-derived ECs and SMCs	human	Seeding cells onto nano-fibular scaffolds to generate vascular grafts	(198)
	iPSC-derived vascular cells	human	Production of human vascular organoids that showed successful integration into immunodeficient mice and further maturation post-transplant	(199)
	iPSC-derived SMCs	human	Formation of a dense cell which was shaped to a tubular structure as tunica media	(200)

1211

1212 **Table 2:** Recent proteome datasets for smooth muscle and endothelial cells together

1213 with available (open access) PRIDE accession numbers.

1214

Year	**Title / PRIDE accession number**	**Protein Identifications**	**Cell Type(s)**	**Reference (DOI)**
2023	Multi-omics profiling of human endothelial cells from the coronary artery and internal thoracic artery reveals molecular but not functional heterogeneity (PXD037861)	3325	ECs	(238)
2023	Effect of furoxans on smooth muscle cell proliferation by SILAC experiments (PXD043814)	838	SMC	(248)
2023	Smooth Muscle Cell Phenotypic Switch Induced by Cigarette Smoke Condensate (PXD041174)	N/A	SMC	(249)
2022	Heart tissue extracellular vesicles reprogram smooth muscle cell proteome (PXD038482)	N/A	SMC	Unpublished
2023	Anastellin impacts on the processing of extracellular matrix and stimulates the	N/A	SMC (secretome)	(250)

	release of cytokines from smooth muscle cells (PXD033732)			
2022	Characteristics of extracellular vesicles secreted by senescent human vascular smooth muscle cells and its influence on immune cells (PXD030955)	1480	SMC (EVs)	(251)
2022	Proteomic Profiling of Concurrently Isolated Primary Microvascular Endothelial Cells, Pericytes, and Smooth Muscle Cells from Adult Mouse Heart (PXD026673)	4694	ECs, SMCs	(252)
2019	Differentially Expressed Proteins in Primary Endothelial Cells Derived From Patients With Acute Myocardial Infarction *Not publically released*	2246	ECs	(253)

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