Dissertation

Liver X Receptor activation attenuates oxysterol induced inflammatory responses and dysfunction in human fetoplacental endothelial cells

Submitted by

Meekha GEORGE

For the Academic Degree of

Doctor of Philosophy (Ph.D.)

at the

Medical University of Graz

Department of Obstetrics and Gynecology

under the supervision of

Univ. Prof. Dr. Christian WADSACK

2023

Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgment has been made in the text to all other material used. Throughout this thesis and in all related publications, I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz."

Meekha George

August 2023

Disclosures

Part of the thesis has been published in **George M**, Lang M, Gali CC, Babalola JA, Tam-Amersdorfer C, Stracke A, Strobl H, Zimmermann R, Panzenboeck U, Wadsack C. "Liver X Receptor Activation Attenuates Oxysterol-Induced Inflammatory Responses in Fetoplacental Endothelial Cells." Cells. 2023; 12(8):1186. https://doi.org/10.3390/cells12081186 (1).

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George M., Allerkamp H.H., Koshenov Z., Oflaz F., Tam-Amersdorfer C., Rittchen S., Kolesnik T., Fröhlich E., Graier W., Strobl H., Wadsack C., Targeting Liver-X receptors to mitigate oxysterol-induced dysfunction in fetoplacental endothelial cells (Submitted)

Articles published as co-author during PhD:

Strahlhofer-Augsten, M., Schliefsteiner, C., Cvitic, S., **George, M**., Lang-Olip, I., Hirschmugl, B., Marsche, G., Lang, U., Novakovic, B., Saffery, R., Desoye, G., & Wadsack, C. (2022). The Distinct Role of the HDL Receptor SR-BI in Cholesterol Homeostasis of Human Placental Arterial and Venous Endothelial Cells. International journal of molecular sciences, 23(10), 5364. https://doi.org/10.3390/ijms23105364

Baković, P., Kesić, M., Perić, M., Bečeheli, I., Horvatiček, M., **George, M**., Čičin-Šain, L., Desoye, G., Wadsack, C., Panzenboeck, U., & Štefulj, J. (2021). Differential Serotonin Uptake Mechanisms at the Human Maternal-Fetal Interface. International journal of molecular sciences, 22(15), 7807. https://doi.org/10.3390/ijms22157807

Babalola, J. A., Lang, M., **George, M**., Stracke, A., Tam-Amersdorfer, C., Itxaso, I., Lucija, D., Tadic, J., Schilcher, I., Loeffler, T., Flunkert, S., Prokesch, M., Leitinger, G., Lass, A., Hutter-Paier, B., Panzenboeck, U., & Hoefler, G. (2023). Astaxanthin enhances autophagy, amyloid beta clearance and exerts anti-inflammatory effects in in vitro models of Alzheimer's disease-related blood brain barrier dysfunction and inflammation. Brain research, 1819, 148518. https://doi.org/10.1016/j.brainres.2023.148518

Co-authors contribution:

All co-authors have agreed to the use of the data in the dissertation

Otto-Loewi Research Center for Vascular Biology, Immunology and Inflammation, Division of Immunology, Medical University of Graz, Austria.

Magdalena Lang, Anika Stracke, Chaitanya Chakravarthi Gali, and Christina Angelika Passegger - contributed to the lab methodology

Carmen Tam–Amersdorfer helped to perform immunofluorescence images

Sonja Rittchen supported the setup of endothelial barrier experiments

Herbert Strobl supported to draft the publications

Gottfried Schatz Research Center, Department of Molecular Biology and Biochemistry, Medical University of Graz, Austria.

Zhanat Koshenov, Furkan Enes Oflaz, and Wolfgang Graier supported to perform mitochondrial membrane potential and calcium flux measurements

Core facility imaging, Center for Medical Research, Medical University of Graz, Austria

Eleonore Fröhlich and Tatjana Kolesnik performed Atomic Force microscopy and data analysis

Institute of Molecular Biosciences, University of Graz, Austria

Robert Zimmermann helped with the overall concept of the thesis

Diagnostic and Research Institute of Pathology, Medical University of Graz, Austria

Joshua Adekunle Babalola supported to perform RNA isolation and qPCR experiments

Acknowledgments

This doctoral thesis was conducted as part of the PhD Doctoral Program - Inflammatory Disorders in Pregnancy (DP-iDP), supported by the Austrian Science Fund FWF (DOC 31-B26) and the Medical University of Graz, Austria.

First and foremost, I would like to express my deepest gratitude to **Prof. Christian Wadsack**, my supervisor, for his unwavering support and guidance throughout my PhD journey. His invaluable advice and feedback have greatly enhanced the quality of my scientific work in all aspects. I sincerely express my gratitude for his willingness to take over my project after the demise of my former PhD supervisor **Prof. Ute Panzenboeck**. I would also like to take this opportunity to express my sincere gratitude to Ute who entrusted me with her proposed project. I am certain that you are looking down on me from heaven with joy at my achievement. Finally, I am thankful to my thesis committee members, **Prof. Herbert Strobl** and **Prof. Robert Zimmermann**, for their constant support and input during my PhD journey.

The members of the Institute of Immunology and Pathophysiology and the Department of Obstetrics and Gynecology have contributed immensely to my professional and personal time during this PhD journey. I am very obliged for their scientific help and contribution to the thesis, but more importantly for their friendship and support. In particular, I want to thank my colleagues **Magdalena, Joshua, Sally, Christina, Carmen, Anika, Nassim** and **Chaitanya** for making my life in the lab easy, productive and interesting. I am especially thankful for the immense help I received from my collaborators **Zhanat Koshenov, Furkan Enes Oflaz, Tatjana Kolesnik** and **Sonja Rittchen**. This PhD work would not have been accomplished without their help and support.

I want to thank my family, especially my parents**, Mini and George, for being supportive as always,** even in my tough times. Their endless and unconditional love is the biggest support for me to pursue my dreams and career far away from home, in another country. I am also grateful to my brother, **Nohesh**, who has always been a source of courage and love. I also want to thank my dear friends who made my stay in Graz a wonderful experience. Last but not least, I am extremely thankful to my husband **Anu Sebastian** who quit his Job in Abu Dhabi and moved to Graz after our marriage and constantly showered me with love, warmth and support. Thanks for cheering me up and being with me as my pillar of strength. Words cannot express my gratitude and love towards you.

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Abbreviations

	7β-OHC	7β-hydroxycholesterol
	7-ketoC	7-ketocholesterol
	24-OHC	24-hydroxycholesterol
	27-OHC	27-hydroxycholesterol
A	ABCA1	ATP binding cassette transporter A1
	ABCG1	ATP binding cassette transporter G1
	AFM	Atomic force microscopy
B	BCA	Bicinchoninic acid
	BSA	Bovine serum albumin
C	CFSE	Carboxyfluorescein succinimidyl ester
	CYP	Cytochrome
D	DMSO	Dimethyl sulfoxide
E	ECIS	Electric Cell-substrate Impedance Sensing
	ERK	Extracellular signal-regulated kinase
	eNOS	Endothelial nitric oxide synthase
F	FCCP	Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone
	FCS	Fetal calf serum
	FITC	Fluorescein isothiocyanate
	FpAEC	Fetoplacental arterial endothelial cells
	FpEC	Fetoplacental endothelial cells
	FpVEC	Fetoplacental venous endothelial cells
G	GDM	Gestational diabetes mellitus
	GLUT4	Glucose transporter type 4
	GPCR	G-protein coupled receptor
	GSK	GSK3033
H	HBSS	Hanks balanced salt solution
	HDL	High density lipoproteins
	HPRT1	Hypoxanthine phosphoribosyltransferase 1
	HRP	Horseradish peroxidase
I	ICAM-1	Intercellular adhesion molecule 1
	IL	Interleukin
	IUGR	Intrauterine growth restriction
J	JNK	Jun N-terminal kinase
L	LDH	Lactate dehydrogenase
	LDL	Low-density lipoproteins
	LPS	Lipopolysaccharide
	LXR	Liver-X receptor
	LXRE	LXR response element
M	MAPK	Mitogen-activated protein kinase
	MMP	Matrix metalloproteinase
	MyD88	Myeloid differentiation primary response 88
N	NFκB	Nuclear factor-kappa B
	NO	Nitric oxide

O	OGTT	Oral glucose tolerance test
	OxLDL	Oxidized low-density lipoproteins
P	PBS	Phosphate buffer saline
	PE	Preeclampsia
	PFA	Paraformaldehyde
	PLTP	Phospholipid transfer protein
Q	qPCR	Quantitative real-time polymerase chain reaction
R	RNA	Ribonucleic acid
	ROS	Reactive oxygen species
	RT	Reverse transcription
	RXR	Retinoid X receptors
S	SD	Standard deviation
	SEM	Standard error mean
	SDS PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
	SRB-1	Scavenger receptor class B type 1
	SREBP	Sterol regulatory element binding proteins
	SUMO	Small Ubiquitin-like Modifier
T	TBST	Tris-buffered saline with Tween20
	TLR	Toll-like Receptor
	TMRM	Tetramethylrhodamine methyl ester
	TNF-α	Tumor necrosis factor-α
	TO	T0901319
V	VCAM-1	Vascular cell adhesion Molecule 1
	VE-cadherin	Vascular-endothelial cadherin
	VEGF	Vascular endothelial growth factor
	VWF	Von Willebrand factor

Abstract

Oxysterols are a family of sterols formed mostly by enzymatic cholesterol oxidation. Oxysterols can also be formed via non-enzymatic reactions both in vivo and ex vivo. Their systemic levels are found elevated in inflammatory pregnancy disorders such as gestational diabetes mellitus (GDM) and preeclampsia (PE). Oxysterols act through various intracellular and extracellular receptors, play diverse roles in regulating various physiological processes, and can have implications for human health and disease. Pregnancy disorders are often associated with chronic inflammation accompanied by altered inflammatory profiles in the mother, placenta, and fetus. Elevated levels of two oxysterols, namely, 7-ketocholesterol (7-ketoC) and 7β-hydroxycholesterol (7β-OHC), were observed in the cord blood of GDM offspring as well as in fetoplacental endothelial cells (fpEC). The main objective of this thesis was to investigate the impact of 7-ketoC and 7β-OHC on fetoplacental endothelial cells (fpEC). Subsequently, the effects of oxysterols on cellular mechanisms, such as endothelial dysfunction and their role in inflammation, were examined. Primary fpEC in culture, treated with 7-ketoC or 7β-OHC induced the activation of mitogen associated protein kinase (MAPK) and nuclear factor kappa B (NFκB) signaling which subsequently activated pro-inflammatory cytokines such as interleukin 6 (IL-6) and interleukin 8 (IL-8), and intercellular cell adhesion molecule-1 (ICAM-1). Furthermore, oxysterols elevatedintracellular calcium mobilization and reduced barrier impermeability by disrupting the VE-cadherin adherens junction, resulting in the formation of actin stress fibers and consequently enhancing the stiffness of the plasma membrane. Oxysterols promoted reactive oxygen species (ROS) generation, THP-1 monocyte attachment and disrupted mitochondrial bioenergetics. We investigated the impact of the liver X receptor (LXR) on oxysterol-induced altered cellular responses, whose activation is known to be associated with repression of inflammation and stabilization of endothelial barrier integrity. Treatment with LXR synthetic agonist dampened oxysterol-induced activation of pro-inflammatory signaling pathways, expression of cytokines and cell adhesion molecules. Additionally, LXR activation protected fpEC from the detrimental effects of oxysterols, including the loss of barrier integrity, the generation of reactive oxygen species (ROS), and the attachment of monocytes. Probucol, a known efflux activity inhibitor of LXR target gene ATP-binding cassette transporter 1 (ABCA1) protein, antagonized the anti-inflammatory effects of LXR agonist against oxysterols. This suggests the potential involvement of ABCA1 in LXR-mediated repression of inflammatory signaling in fpEC. Toll Like Signaling (TLR) 4 inhibition

in fpEC abolished oxysterol-induced MAPK and NFκB inflammatory signaling cascade and further downstream responses. Furthermore, inhibition of LXR activation abrogated the vasoprotective effects, suggesting an essential role of LXR in the maintenance of endothelial barrier functions. Collectively, our results indicate that 7-ketoC and 7β-OHC play a role in promoting placental inflammation by triggering the TLR4 signaling pathway and causing endothelial dysfunction, as observed in severe pathophysiological pregnancies. LXR activation has the potential to rescue fpEC from oxysterol-induced inflammation and barrier dysfunction, making them a potential target for the treatment of inflammatory diseases.

Zusammenfassung

Oxysterole sind eine Familie von Sterolen, die hauptsächlich durch enzymatische Cholesterinoxidation gebildet werden. Oxysterole können auch durch nicht-enzymatische Reaktionen sowohl in vivo als auch ex vivo gebildet werden. Ihre systemischen Spiegel sind bei entzündlichen Schwangerschaftserkrankungen wie Gestationsdiabetes mellitus (GDM) und Präeklampsie (PE) erhöht. Oxysterole wirken über verschiedene intrazelluläre und extrazelluläre Rezeptoren, spielen eine vielfältige Rolle bei der Regulierung verschiedener physiologischer Prozesse und können Auswirkungen auf die menschliche Gesundheit und Krankheit haben. Schwangerschaftsstörungen sind häufig mit chronischen Entzündungen verbunden, die mit veränderten Entzündungsprofilen bei der Mutter, der Plazenta und dem Fötus einhergehen. Erhöhte Konzentrationen von zwei Oxysterolen, nämlich 7-Ketocholesterin (7-KetoC) und 7β-Hydroxycholesterin (7β-OHC), wurden im Nabelschnurblut von GDM-Nachkommen sowie in fetoplazentaren Endothelzellen (fpEC) beobachtet. Das Hauptziel dieser Arbeit war es, die Auswirkungen von 7-KetoC und 7β-OHC auf fetoplazentare Endothelzellen (fpEC) zu untersuchen. Anschließend wurden die möglichen Auswirkungen von Oxysterolen auf zelluläre Mechanismen wie endotheliale Dysfunktion und ihre Rolle bei Entzündungen untersucht. Primäre fpEC in Kultur, die mit 7-KetoC oder 7β-OHC behandelt wurden, induzierten die Aktivierung der mitogenassoziierten Proteinkinase (MAPK) und des Nuclear Factor Kappa B (NFκB) Signals, die anschließend pro-inflammatorische Zytokine wie Interleukin 6 (IL-6) und Interleukin 8 (IL-8) sowie das interzelluläre Zelladhäsionsmolekül-1 (ICAM-1) aktivierten. Des Weiteren erhöhten Oxysterole die intrazelluläre Calciummobilisierung und verringerten die Barriereintegrität, indem sie die VE-Cadherin-Adherensverbindungen störten, was zur Bildung von Aktin-Stressfasern führte und folglich die Steifigkeit der Plasmamembran erhöhte. Oxysterole förderten die Bildung reaktiver Sauerstoffspezies (ROS), die Anheftung von THP-1-Monozyten und störten die mitochondriale Bioenergetik. Wir untersuchten die Auswirkungen des Leber-X-Rezeptors (LXR) auf die durch Oxysterole ausgelösten veränderten zellulären Reaktionen, dessen Aktivierung bekanntermaßen mit der Unterdrückung von Entzündungen und der Stabilisierung der Integrität der Endothelbarriere verbunden ist. Die Behandlung mit einem synthetischen LXR-Agonisten dämpfte die durch Oxysterol ausgelöste Aktivierung proinflammatorischer Signalwege, die Expression von Zytokinen und Zelladhäsionsmolekülen. Darüber hinaus schützte die LXR-Aktivierung fpEC vor den schädlichen Auswirkungen von Oxysterolen, einschließlich des

Verlusts der Barriereintegrität, der Bildung reaktiver Sauerstoffspezies (ROS) und der Anheftung von Monozyten. Probucol, ein bekannter Inhibitor der Efflux-Aktivität des LXR-Zielgens ATP-binding cassette transporter 1 (ABCA1) Protein, hemmte die entzündungshemmende Wirkung des LXR-Agonisten gegen Oxysterole. Dies deutet auf eine mögliche Beteiligung von ABCA1 an der LXR-vermittelten Unterdrückung von Entzündungssignalen in fpEC hin. Die Hemmung von Toll Like Signaling (TLR) 4 in fpEC hob die durch Oxysterole ausgelöste MAPK- und NFκB-Signalkaskade und weitere nachgeschaltete Reaktionen auf. Darüber hinaus hob die Hemmung der LXR-Aktivierung die vasoprotektiven Effekte auf, was auf eine wesentliche Rolle von LXR bei der Aufrechterhaltung der endothelialen Barrierefunktionen hindeutet. Insgesamt deuten unsere Ergebnisse darauf hin, dass 7-KetoC und 7β-OHC über die Aktivierung der TLR4-Signalkaskade zur Plazentaentzündung und zur endothelialen Dysfunktion beitragen, wie sie bei schweren pathophysiologischen Schwangerschaften beschrieben wurde. Die LXR-Aktivierung hat das Potenzial, Oxysterol-induzierte Entzündungen und Barrieredysfunktionen in fpEC zu dämpfen, was sie zu einem potenziellen Ziel für die Behandlung von Entzündungskrankheiten macht.

1. Introduction

1.1 The Human Placenta – Structure and Function

The placenta is a well-vascularized, unique organ that grows in the uterus during pregnancy and acts as an interphase between mother and fetus. It has a crucial role in proper fetal growth and development as it transports nutrients to the growing fetus and removes waste products from the fetal circulation (2). The human placenta consists of a fetal side called the chorionic plate, which is connected to the fetus by the umbilical cord that extends from the chorionic plate, and a maternal side called the basal plate (Figure 1) (3). The region between the fetal and maternal sides is the intervillous space, which acts as the fetal-maternal interphase (4). The intervillous space is lined by the multinucleated syncytium (5). The intervillous space is the main site of exchange of nutrients, water, gases, and waste between the maternal and fetal circulations (6) (Figure 1). The placenta has two separate circulatory systems known as the maternal-placental and fetal-placental blood circulations. For efficient exchange between the circulations, chorionic villi occupy much of the placenta and develop into branching tree-like structures to maximize surface area contact with the maternal blood. Through the maternal placental circulation, the maternal blood reaches the intervillous space where it surrounds the villi. Deoxygenated fetal blood is carried in the umbilical arteries and reaches the chorionic villi. After an exchange of nutrients and oxygen, the umbilical vein returns the oxygenated blood to the fetal systemic circulation. The two circulations are in close proximity but are physically separated from each other. In principle, these two circulations never mix in a normal physiological state (7). The placenta acts as a physical barrier, protecting the growing fetus from toxic substances for its proper development (8).

The epithelial-structured barrier that separates maternal from fetal blood is called syncytium (9). The syncytium is delimited by mesenchymal stromal cells consisting of placental macrophages called Hofbauer cells and fibroblasts (10). The architecture of the villous vasculature consists of endothelial cells, vascular smooth muscle cells and pericytes (11). Efficient maintenance of the structure and function of each component of the placenta is essential for the well-being and development of the fetus in the womb. The placenta also senses changes in the gestational environment and plays a central role in modulating maternal physiology to support fetal development (12). Disruption of placental homeostasis can lead to

several pregnancy disorders, including gestational diabetes mellitus (GDM), Preeclampsia (PE) and intrauterine growth restriction (IUGR) (13).

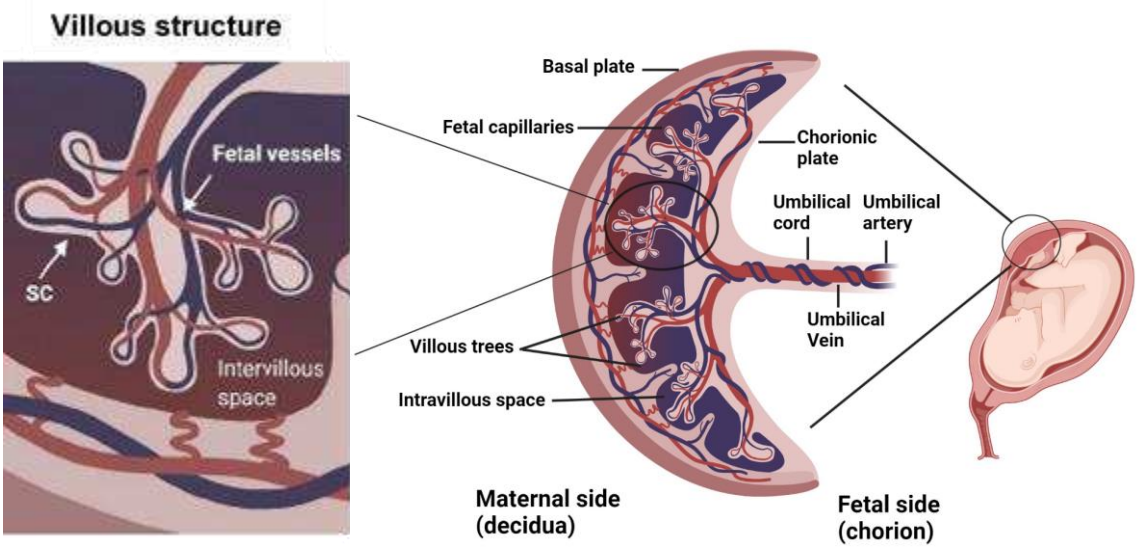


Figure 1: Schematic structure of the human placenta. The fetal side of the placenta is referred to as the chorionic plate. The umbilical vein (red) carries nutrient enriched and oxygenated blood to the fetus. From the fetus back to the placental vasculature, deoxygenated blood for gas and nutrient exchange is carried back by two umbilical arteries (blue). The exchange area between mother and fetus in the placenta is structured in villous trees, which are surrounded by a layer called syncytium (SC). The SC is directly in contact with maternal blood and maintains physical separation between the maternal and fetal circulations. The space between the villi is called the intervillous space, in which the nutrient and gas exchange takes place. Fetal vessels are embedded in each branch of the villous tree, facilitating the exchange of nutrients and gas. The figure has been created with BioRender.com.

1.1.1 Human fetoplacental endothelial cells (fpEC)

The chorionic plate arteries and veins originate from the umbilical cord insertion and cross the fetal surface of the placenta until they penetrate the villi (14). The chorionic arteries arise from the umbilical artery, divide into two smaller arteries and they always cross the chorionic vein (15). This complex vascular network within the placenta plays a crucial role in vascular physiology by regulating vasomotor tone and blood flow to the fetus. The complex branching pattern of the chorionic arteries allows precise control of blood flow, ensuring

optimal oxygen and nutrient supply to the developing fetus (16). The chorionic vasculature of the placenta responds to several vasoactive compounds, including angiotensin II (AngII), prostaglandins, endothelin, and nitric oxide (NO), which play a role in regulating placental vascular resistance. Imbalances in the production of these vasoactive mediators in the placenta have been implicated in the development of pregnancy-associated syndromes such as PE and GDM (17,18). The mechanisms responsible for placental pathology are not yet fully understood, but it is widely recognized that the dysfunction of the fetoplacental endothelium plays a significant role (19).

Primary placental endothelial cells isolated from chorionic plate arteries or veins are excellent in vitro models to study vascular defects in the placenta under physiological or pathophysiological conditions. Fetoplacental endothelial cells from chorionic venous vessels (fpVEC) are spindle-shaped and predominantly express transporter genes, suggesting a role in nutrient transport to the fetus. FpVEC have increased expression of developmental-associated genes such as gremlin, mesenchyme homeobox 2, and stem cell protein DSC54 and display an increased potential to differentiate into osteoblasts and adipocytes compared to fpAEC. Whereas endothelial cells from placental arteries (fpAEC) predominantly express genes associated with placental vascularization and angiogenesis, such as vascular endothelial growth factor A (VEGF), suggesting their potential involvement in the formation and maintenance of the placental vasculature (20). Furthermore, genes involved in cholesterol and lipid homeostasis such as liver-X receptor β (LXRβ), phospholipid transfer protein (PLTP), scavenger receptor class B type 1 (SRB1), ATP-binding cassette (ABC) transporters ABCA1 and ABCG1 are greater expressed in fpAEC than in fpVEC (21,22). The morphological integrity and phenotypic heterogeneity of isolated fetoplacental endothelial cells (fpEC) in cell culture are maintained up to several passages with no significant loss of functional or morphological integrity (20). Thus, fpEC are considered a well-established in vitro model to study the function and development of fetoplacental endothelium. The purity of isolated fpEC is assessed by staining for endothelial markers Ulex europaeus agglutinin I (UEA-I) lectin, von Willebrand factor (vWF), and DiI-Ac-LDL uptake (20).

Integrity of intercellular junctions is a significant determinant of permeability of the endothelium, and the VE-cadherin-based is thought to be particularly important. VE-cadherin is required for maintaining a restrictive endothelial barrier in the human placenta (23). The fpEC in the placental barrier express a wide range of molecular transporters for the uptake

as well as for the transfer of substances into the fetal circulation (24). During pregnancy, the placental vasculature undergoes extensive remodeling through endothelial cell proliferation and elongation. Any vascular defects due to placental endothelial cell dysfunction can contribute to developing disorders such as IUGR and PE (25).

1.2 Placental endothelial dysfunction in pregnancy disorders

The endothelium plays a vital role in the regulation of vasomotor tone, platelet aggregation, blood coagulation, blood fluidity, and pressure. Therefore, endothelial cells serve as critical regulators of inflammation, vasculogenesis and angiogenesis (26–28). The endothelium also functions as an endocrine organ, secreting paracrine-signaling molecules such as nitric oxide and prostaglandins. Nitric oxide (NO), synthesized by endothelial nitric oxide synthase (eNOS), is the major contributor to endothelial function (29). A reduction in the bioavailability of vasodilators, mainly NO, and an increase in vasoconstrictors leads to endothelial dysfunction (30). As a result, the endothelium shifts towards a pro-thrombic and pro-inflammatory state, which may contribute to the development and progression of atherosclerosis, cancer-associated angiogenesis, vascular permeability, and infectious diseases (31). Dysfunctional endothelium might increase the risk of insulin resistance and diabetes (32). The endothelium is affected by metabolic disorders through loss of barrier integrity and exposure to inflammation (33).

Adaptation of the maternal vasculature during pregnancy to increase blood flow through the uteroplacental unit to meet the needs of the developing fetus is essential for a healthy pregnancy. Failure of maternal and placental vasculature to adapt appropriately may result in pregnancy disorders, which in turn can affect fetal growth and development (25). One type of adaptation is the increased production of vasodilators in the systemic circulation to maintain blood pressure and adequate uteroplacental blood flow (34). Proper maintenance of the barrier integrity is also very important for the appropriate compartmentalization of the vascular and interstitial spaces, allowing for controlled trafficking of molecules and preventing unwanted leakage of substances (35,36).

Throughout pregnancy, the placental vasculature provides a barrier that regulates the exchange of substances between the mother and the growing fetus (37). The intercellular adhesion protein vascular endothelial cadherin (VE-cadherin) is the principal regulator of cell adhesion, forming the adherens junction that controls vascular permeability (38). Inadequate

blood supply to the fetus due to impaired placental vascular function can have severe consequences during pregnancy. Placental insufficiency, also known as placental dysfunction, is a rare but significant complication that can occur when the placenta fails to develop properly or is damaged, resulting in a reduced blood supply from the mother to the growing fetus. Consequently, disturbances in the structure, integrity and function of the placental vasculature during pregnancy have been implicated in a variety of pregnancy complications (19,39).

1.1.2 Gestational Diabetes Mellitus

Gestational diabetes mellitus is a complication in pregnancy that occurs in approximately 15% of pregnancies (40). GDM is characterized by elevated blood glucose levels during pregnancy and is diagnosed by an oral glucose tolerance test (OGTT) at 24 weeks of gestation (41,42). GDM increases the risk of adverse maternal and neonatal outcomes, such as the risk of developing type 2 diabetes after pregnancy, increased neonatal obesity, macrosomia and preterm birth (43,44). Increased secretion of placental growth hormone and human placental lactogen (hPL) contributes to insulin resistance and inadequate pancreatic cell compensation contributes to the development of GDM (45,46). Maternal glucose crosses the placental barrier, but maternal insulin does not, provoking fetal hyperglycemia with adverse long-term consequences for the child by increasing the risk of developing metabolic and cardiovascular disease (47,48).

GDM induces dysfunction of the fetoplacental vasculature (49). Multiple factors have been identified that contribute to this pathophysiology. GDM is often accompanied by elevated inflammatory profiles in the maternal and fetal circulation (50). Additionally, pro-inflammatory cytokines, cell adhesion molecules such as ICAM1, VCAM1 and E-selectin are systemically elevated, which together contribute to endothelial cell activation and leukocyte adhesion and subsequent development of GDM-associated endothelial dysfunction (51,52). GDM is linked to the increased generation of ROS, thereby increasing the oxidative stress throughout the pregnancy (53). The bioavailability of NO produced to effectively maintain vascular tone and vascular function to adapt to conditions of GDM is reduced by the reaction with ROS (54). The interaction of NO with the oxygen radical O_2^- generates peroxynitrite ($ONOO^-$), a highly reactive radical molecule that can cause protein, lipid and DNA damage (55). In one study, GDM patients had reduced flow-mediated dilation (FMD), which measures the change in arterial diameter and is used as a useful tool to determine proper vascular function. In addition,

GDM patients had decreased serum NO levels with increased endothelin-1, a potent vasoactive peptide implicated in endothelial dysfunction (56).

1.1.3 Preeclampsia

Preeclampsia is a pregnancy complication that affects 5-10% of the pregnancies diagnosed after 20 weeks of gestation (57). It is characterized by hypertension and proteinuria (58). PE can affect different organs in the mother's body, including the liver and kidney and is also detrimental to the growth and development of the fetus. Women usually recover from the condition after childbirth, but they are predisposed to develop cardiovascular diseases in their later life (59).

In PE, inadequate uteroplacental perfusion leads to the dysfunction of the maternal vascular endothelium as a consequence of increased systemic resistance which in turn leads to a lower perfusion of maternal organs (60,61). During the first trimester of gestation, the extravillous trophoblast cells of fetal origin proliferate and invade the maternal decidua while the maternal spiral arteries transform into high-capacity, low-resistance vessels from low-capacity, high-resistance vessels ensuring proper placental perfusion (62). In conditions of PE, the invasion of trophoblasts is impaired followed by a significant decrease in the remodeling of spiral arteries and poor placental perfusion (63,64). This affects the oxygen and nutritional status of the placenta and leads to placental endothelial dysfunction. Consequently, interrupted arterial blood flow generates repeated ischemia which creates oxidative stress (65). Excessive ROS production disrupts mitochondrial bioenergetics, induces eNOS dysfunction and activates matrix metalloproteinase and placental inflammasome. Consequently, inflammation is triggered by the activation of NFκB-mediated transcription of cytokines such as IL6, IL8, TNFα and cell adhesion molecules such as ICAM1 and VCAM1 (66–68). Endothelial cells from the human umbilical vein (HUVECs) in PE exhibit reduced expression and activity of eNOS (69). Additionally, there was evidence of inhibition of the nitric oxide (NO) pathway in the fetal-placental vasculature of preeclamptic placentas (70).

1.3 Oxysterols

Oxysterols are bioactive oxidized cholesterol derivatives formed as intermediates in bile acid synthesis or steroid hormone biosynthesis (71). They are sometimes the end products of cholesterol metabolism (72). Oxysterols were considered mere byproducts in the cholesterol excretion pathway for a long time. These products are now fully considered bioactive lipids after the discovery of their ability to bind to several cellular receptors and initiate a plethora of signaling pathways (73). Oxysterols are generated in cells mainly through two pathways. Firstly, they can be produced by enzymatic reactions in which cytochrome 450 (CYP) is involved (e.g. 24-hydroxycholesterol (24-OHC), 27-hydroxycholesterol (27-OHC), 25-hydroxycholesterol (25-OHC). Secondly, non-enzymatic reactions triggered by reactive oxygen species (ROS) form differently composed oxysterols (7α-hydroxycholesterol (7α-OHC) 7-ketocholesterol (7-ketoC), 7β-hydroxycholesterol (7β-OHC) (74). Oxysterols generated through different pathways are depicted in Figure 2. Oxysterols are also obtained from the dietary intake of processed foods rich in cholesterol. All cholesterol oxidation products are absorbed in the gut (75).

Oxysterols are generated in numerous tissues due to the expression of CYP and also in conditions of oxidative stress (73). The introduction of oxygen to cholesterol alters its physical properties, thereby making it easier to rapidly cross the plasma membrane, especially the side chain oxidized cholesterols (76). Cholesterol synthesized in the brain is removed from the central nervous system in the form of oxysterols. This is because of its ability to pass through the blood-brain barrier (BBB). In the human brain, 24(S)-OHC, 7-ketoC and 7β-OHC have been shown to cross the BBB to the periphery (77).

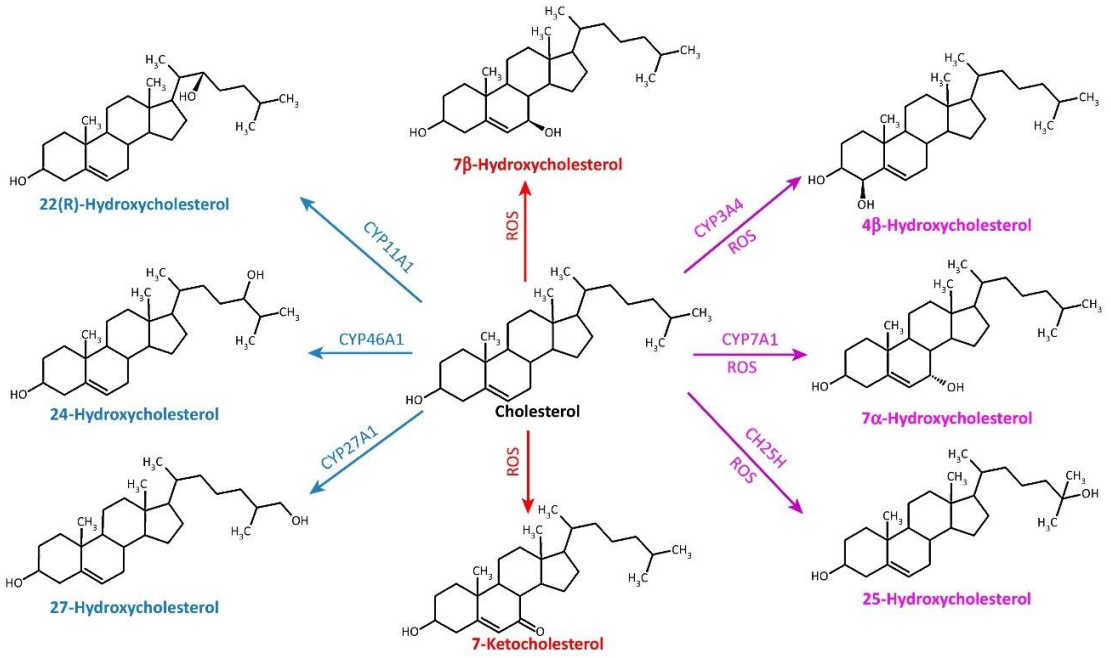


Figure 2. Generation of oxysterols from cholesterol oxidation via enzymatic and non-enzymatic reactions. The enzymatic pathways involve the cytochrome P450 (CYP) family and cholesterol-25-hydroxylase (CH25H). Enzymatic reactions produce side-chain oxidized oxysterols (blue) and non-enzymatic oxidation by ROS give rise to ring oxysterols (red). Some oxysterols are produced by both enzymatic and non-enzymatic reactions (purple). Reproduced from Kloudova et al. 2017 with permission of Trends in Endocrinology & Metabolism (78).

1.3.1 Role of oxysterols in inflammatory disorders

In healthy individuals, the plasma concentrations of oxysterols are very low, varying between mid- to high- nanogram/milliliter range (79,80). Although found at very low concentrations in vivo, these metabolites exert a significant impact on both health and disease, particularly in the development and progression of cardiovascular and inflammatory diseases (81). Several studies documented a rise in oxysterol concentration in the plasma of patients with different metabolic diseases such as obesity, type 2 diabetes and other cardiovascular diseases. ROS-derived oxysterols such as 7-ketoC and 7β-OHC are often found associated with those disorders (82,83). In hyperlipidemic patients, 27-OHC and 7-ketoC are further increased compared to diabetic patients (82). Several studies show evidence that increased plasma oxysterols can be a risk factor for heart disease (84). They are also associated with inducing apoptosis and necrosis in a variety of cell types at higher concentrations. 7-ketocholesterol and

7β-hydroxycholesterol are mostly cytotoxic in different cell types (85). Some studies have indicated that oxysterols enter retinal pigmentary epithelial cells and induce excess ROS production, subsequently causing oxidative stress (86).

Inflammation is a major risk factor for the progression and complications of chronic diseases. ROS amplify inflammation, which in turn further induces ROS generation, eventually giving rise to a vicious cycle that sustains the inflammatory reactions that become detrimental (87). Oxysterols have been implicated in a range of inflammatory diseases, encompassing both infectious and non-communicable diseases (81). Several oxysterols are identified as potent inducers of inflammatory mediators, including cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), monocyte chemotactic protein-1 (MCP-1) and monocyte inflammatory protein-1β (MIP-1β), and cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (88). Oxysterols have been shown to enhance matrix metalloprotease-9 (MMP-9) levels in macrophage lineages through the induction of NADPH oxidase 2 (Nox2) activity, which in turn enhances ROS production. They are implicated in the pathogenesis of inflammatory bowel diseases, retinal degenerative and neurodegenerative diseases like multiple sclerosis, Alzheimer's and Parkinson's disease (87). Recent studies reveal the potential of oxysterols in modulating cancerous conditions by influencing cell proliferation. In breast cancer models in vitro, 25- and 27-Hydroxycholesterol enhance the transcription of target genes of estrogen receptors, suggesting a potential role in resistance to hormonal therapy. Oxysterols are also found to induce DNA damage, contributing to the development of cancer if the damage is not repaired by the cellular repair machinery (78).

1.3.2 Receptors of oxysterols

Oxysterols can bind to numerous receptors and non-receptor proteins such as lipid transporters. The nuclear receptors oxysterols can bind to are liver X receptors (LXRα and LXRβ), retinoid X receptors (RXRs), estrogen receptor α (ERα) and the Retinoid acid receptor-related orphan receptors (RORs). Oxysterols also bind to several transmembrane receptors including the Epstein-Barr virus-induced gene 2 (EBI2; or GPR183), G-protein-coupled receptors (GPCRs), CXC motif chemokine receptor 2/Interleukin 8 receptor (CXCR2), Smoothened (SMO), and the ionotropic glutamate receptor N-methyl-d-aspartate receptor

(NMDAR). Furthermore, they can bind to cytoplasmic lipid transporter proteins ORP (OSBP-related protein), OSBP (oxysterol-binding proteins), and StarD (Steroidogenic Acute Regulatory (StAR)-related lipid transfer domain proteins). Some oxysterols can interact with the NPC1 (Niemann Pick C1) protein involved in intracellular cholesterol transport. This variety of receptors or non-receptor proteins activated or antagonized by oxysterols explains the large array of effects described for these bioactive lipids in cholesterol and glucose metabolism, as well as in inflammation (73).

LXR activation by oxysterols leads to the upregulation of several cholesterol transporters for intracellular cholesterol homeostasis, enzymes in de novo lipogenesis pathways for lipid homeostasis and gluconeogenic enzymes for glucose homeostasis (89). Activation of ERα by oxysterols is involved in several pathophysiological processes such as osteoporosis, cancer, or cardiovascular diseases (90). It has been proposed that oxysterols can activate GPCRs such as EBI2 or CXCR2 and regulate immune cell migration (91). Oxysterols, particularly 25-OHC are shown to activate smoothened receptor which is implicated in the hedgehog signaling pathway involved in the regulation of embryonic development, cellular homeostasis, and cancer (92). Besides LXRs, oxysterols are involved in cholesterol homeostasis by interacting with insulin induced gene (INSIG), enabling its binding with SREBP cleavage-activating protein (SCAP), preventing cholesterol synthesis under the conditions of high cellular cholesterol (or oxysterols) (93). In placental trophoblasts, oxysterols are shown to activate Toll like receptor (TLR)4 signaling and subsequently induce inflammatory cytokines (94). With the development of research on oxysterol and advanced approaches in cell culture, we can expect the discovery of a plethora of signaling pathways through their cellular receptor regimes such as differentiation, development, inflammation and immunity. The currently known major functions of oxysterols are depicted in Figure 3.

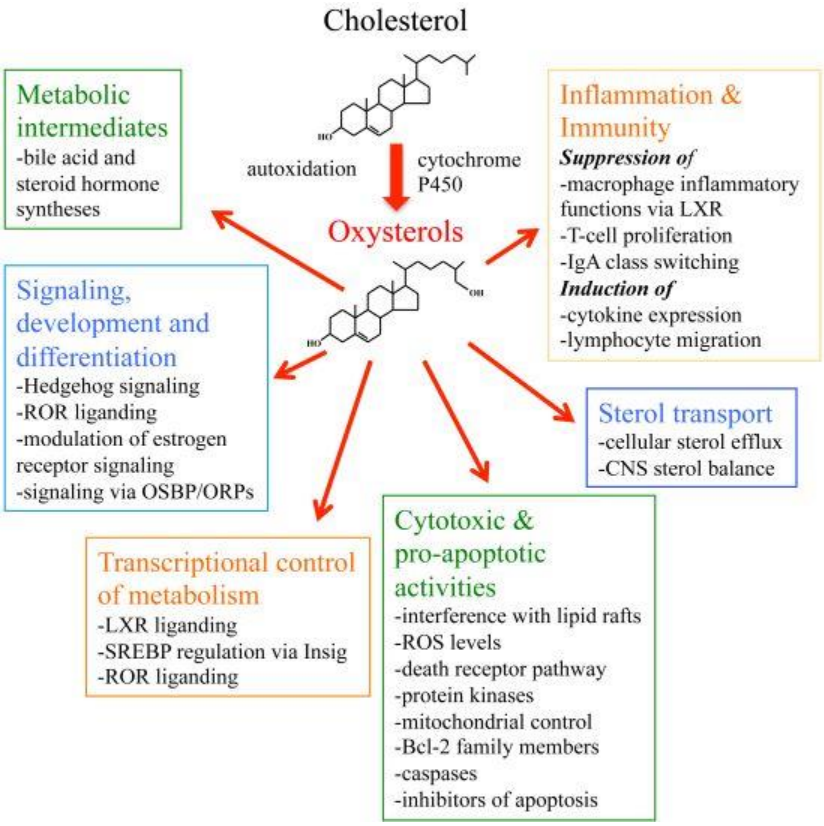


Figure 3: Schematic presentation summarizing the major cellular effectors and functions of oxysterols. ROR, retinoic acid receptor-related orphan receptor; OSBP, oxysterol-binding protein; ORP, OSBP-related protein; Bcl-2, B-cell lymphoma 2; IgA, immunoglobulin A; CNS, central nervous system. Reproduced from Olkkonen et al. 2012 with permission of Biomolecules (95).

1.3.3 Role of Oxysterols in endothelial dysfunction

Several studies have shown that oxidized low-density lipoproteins (oxLDL) containing oxysterols significantly contribute to endothelial dysfunction by favoring platelet aggregation, reducing NO bioavailability by disrupting the activity of endothelial nitric oxide synthase (eNOS), monocyte differentiation into foam cells, which consequently may lead to atherosclerosis (96,97). Oxysterols incorporate into cell membranes, thereby altering endothelial cell membrane fluidity and stiffness and play a major role in dyslipidemia-induced endothelial dysfunction (98,99). The effects on endothelial stiffness have been particularly demonstrated by specific oxysterols such as 7-ketoC and 7α-hydroxycholesterol (98). Additionally, they trigger oxidative stress by increasing the generation of ROS and disrupting the balance between antioxidants and pro-oxidants, thereby contributing to faster vascular aging

(96). Of note, oxysterols play a role in the migration and proliferation of vascular smooth muscle cells (VSMCs), causing intimal thickening, collagen deposition and calcification with consequent lesion formation (100,101). They are also found to be detrimental to physiological endothelial barrier function by inducing increased vascular permeability and leakage (102). In addition to inflammatory effects, oxysterols exert pro-apoptotic properties in pathological concentration in chronic inflammatory diseases and contribute to cellular dysfunction (87).

1.3.4 Oxysterols in pregnancy and placenta

Placenta is likely to be exposed to higher levels of oxysterols during pregnancy, together with oxidative stress and inflammation (94). In pregnancy conditions associated with oxidative stress or inflammation, such as PE or IUGR, the placenta may be exposed to even higher levels of circulating oxysterols (103,104). A recent study discovered the presence of unusual oxysterols such as 20S-OHC and 22S-OHC in human placenta (105). Offspring from hypercholesterolemic pregnancies have increased circulating oxysterol concentrations compared to the offspring from healthy pregnancies (106). Oxidative stress during pregnancy can produce oxysterols which can enhance MMP-14 expression that proteolytically cleaves membrane-bound endoglin and generates the soluble form of endoglin (sEng) in primary placental endothelial cells. Increased sEng levels significantly contribute to the development of hypertension (107). Studies have described an increased concentration of oxysterols, predominantly those derived from ROS (7-ketoC, 7β-OHC) in fpAEC and cord blood of individuals with GDM compared to healthy counterparts (108). Furthermore, oxysterols inhibit the invasion of first-trimester trophoblasts and syncytialization of term placental cytotrophoblast cells. Elevated levels of oxysterols have been observed in pregnancy conditions accompanied by oxidative stress and inflammation. The concentration of oxysterols detected in atherosclerotic lesions is significantly higher than that in the systemic circulation, and the degree of increase is typically correlated with the severity of the lesions (84,109). Therefore, the concentration of oxysterols in atherotic lesions commonly observed in the spiral arteries of preeclamptic placentas might be increased by several folds compared to its normal plasma levels (110,111). In a study, it was observed that the occurrence of acute atherosis lesions in the placenta, encompassing both the basal plate and chorioamnion, was notably more frequent compared to their occurrence in placental bed biopsies, which included both the decidua and myometrial segment (112).

Oxysterols prompt pro-inflammatory responses in placental trophoblasts through a mechanism dependent on TLR4, involving the activation of NF-κB (94). The findings of these studies demonstrate that systemic pathophysiological oxysterol concentrations in pregnancy interfere with the development and function of the placenta (113). Evidence suggests a possible association between increased levels of oxysterols and pregnancy complications like PE and GDM. Oxysterols have been linked to endothelial dysfunction, compromised placental angiogenesis, and inflammation, commonly observed in these conditions (106,114). A study identified that maternal hypercholesterolemia enhances oxysterol concentration in mothers and their offspring (106). However, additional research is required to establish the relationship between elevated oxysterols and pregnancy disorders (114).

1.4 Toll like Receptor signaling in the placenta

Toll-like receptors are transmembrane proteins that belong to a class of pattern recognition receptors involved in eliciting innate immune responses against pathogens by recognizing conserved molecular patterns present on them, thus representing the first line of defense against infection (115). TLRs are expressed on immune cells such as dendritic cells, leukocytes and macrophages, as well as on non-immune cells such as fibroblasts, epithelial and endothelial cells (115,116). Upon recognition of the foreign particle, the TLR is activated and initiates a cascade of signaling events leading to the transcription of pro-inflammatory cytokines, chemokines, and antimicrobial proteins. NFκB and MAPK proteins mainly mediate the TLR signaling pathways (117). Under conditions of oxidative stress, oxidized lipids, glycolipids and carbohydrates can activate similar inflammatory responses, possibly through the activation of pattern recognition receptors such as TLRs (94,118).

TLR activation is one of the first defense mechanisms used by the host cells to initiate innate and subsequent adaptive immune responses to combat invading pathogens (115). However, persistent TLR signaling in cells could disrupt host immune homeostasis through the constant secretion of inflammatory mediators such as pro-inflammatory cytokines and chemokines (119). TLR senses oxidized phospholipids, initiates signaling in conditions of oxidative stress and promotes inflammation in chronic inflammatory diseases (120). Similarly, oxidized lipids and glycolipids may activate a similar inflammatory response in pregnancy-

induced inflammatory disorders associated with oxidative stress, such as PE and GDM, through the activation of cell surface recognition system (94).

To date, ten members of the TLR have been discovered in humans (TLR 1 to TLR 10) (121). Expression of all the members of the TLR has been observed in the human placenta (122). The presence of these receptors during pregnancy is not constant but shows a clear pattern, with an apparent increase in TLR2 and TLR5 transcripts linked to labor (123). This distinct pattern of TLR expression persists until the end of the second trimester (124). Studies have shown the expression of TLR4 in human placentas obtained from both normal and preterm pregnancies during the second and third trimesters. In addition, TLR4 expression has been detected in the syncytiotrophoblast layer by the third trimester, highlighting the importance of placental cells in effectively combating intrauterine infections (124). TLR signaling can trigger an antiviral response in the placental trophoblasts, which have unique properties to effectively respond to viral infections in the placenta (118).

TLR4 is predominantly located on the cell surface and can recognize lipopolysaccharide (LPS) from Gram-negative bacteria. In contrast, TLR4 located on the endosomal membrane initiates signaling upon encounter with viral envelope glycoproteins (125). TLR4 activation by LPS requires the association of several accessory molecules such as LPS binding protein (LBP), cluster of differentiation 14 (CD14) and myeloid differentiation 2 (MD2) (126,127). The TLR4 and MD2 on the cell surface work together to recognize LPS from Gram-negative bacteria to initiate innate immune responses (126). Upon LPS binding, the complex recruits the cytoplasmic adaptor proteins and forms a cluster with the cytoplasmic domains of TLR4 and those of the adaptors (116). The TLR4 signaling cascade is shown in Figure 4. Apart from LPS, TLR4 can be activated by several other factors such as heat-shock proteins, amyloid beta peptides and oxysterols. However, the exact molecular mechanism of the receptor activation is unknown (128–130).

Activation of TLR4 is the primary pathway responsible for initiating the parasite-induced inflammatory process in the placenta (131). Disruption of TLR4 expression or signaling has been observed in conditions such as preterm birth, PE, and abortion (124). Upregulation of the innate immune response occurs during normal pregnancy via TLR activation. However, excessive activity of TLRs and other immune responses has been implicated in the development of pregnancy complications, including pregnancy-induced

hypertension and PE. Studies suggest that abnormal placentation in PE patients is associated with increased expression of TLRs (132). A recent study showed that TLR4 expression was significantly upregulated in PE placentas compared to normal placentas (133) and increased expression of the TLR4-MyD88-NFκB pathway has been detected in GDM placentas, which is associated with increased local insulin resistance in placental tissue (134). A deep understanding of the mechanisms by which TLR4 contributes to the development of pregnancy pathologies is crucial for the development of potential therapeutic strategies aimed at attenuating the adverse effects of TLR4 dysregulation on maternal and fetal health. Further investigations into the involvement of TLR4 in pregnancy pathologies are needed to provide a deeper understanding of the underlying mechanisms and to pave the way for improving pregnancy outcomes, enhancing maternal well-being, and promoting the healthy development of the fetus.

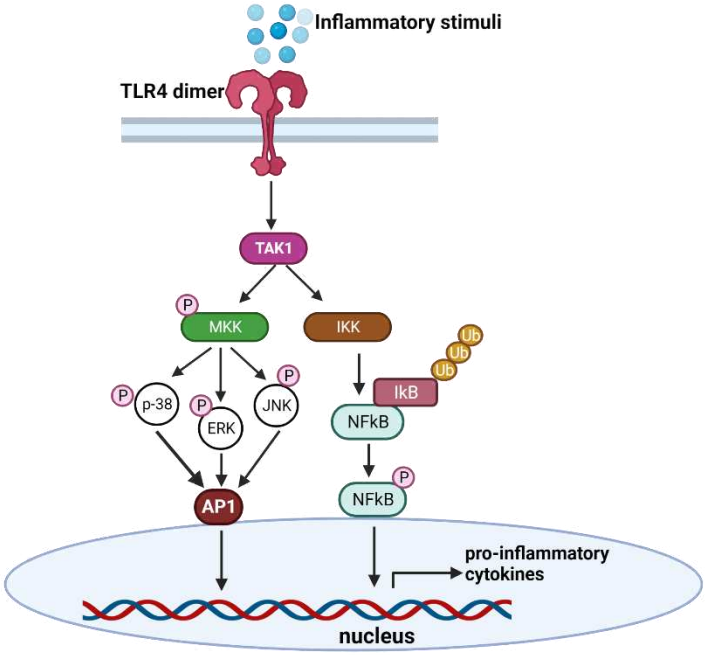


Figure 4: TLR4 pro-inflammatory signaling cascade in immune cells. TLR4 dimerizes upon recognition of inflammatory stimuli like LPS, amyloid beta or HSP at the cell surface and recruits downstream adaptor molecules TRAF6 and TAK1. Activated TAK-1 activates MAP kinase kinase (MKK) by phosphorylation which in turn phosphorylate and activates p38, extracellular signal-regulated kinases (ERK) and JUN N-terminal kinase (JNK) and eventually activates activator protein-1 (AP-1), a transcription factor that promotes the transcription of pro-inflammatory cytokines. TAK1 also phosphorylates IkB kinase complex (IKK) which drives

the proteasomal degradation of IkB by ubiquitination which leads to the activation of nuclear factor kappa B (NFκB) which then translocates to the nuclear and promotes gene transcription of inflammatory cytokines. The figure has been created with BioRender.com.

1.5 Liver -X receptors and its role in the regulation of inflammation

Liver X receptors are ligand-activated transcription factors and are members of the nuclear receptor superfamily of proteins. Two isoforms of LXR have been identified: LXRα (NR1H3) and LXRβ (NR1H2). Both form a heterodimer with retinoid X receptors (RXR) (135) and are reportedly expressed in human placental tissue (136). LXR/RXR heterodimer is inactive under basal conditions by co-repressors and are bound to the response elements of their target gene DNA. When the ligands bind to either of the subunits, the heterodimer undergoes conformational change which leads to the exchange of co-repressors with co-activators. This ultimately leads to the initiation of transcription of target genes, ABCA1, ABCG1 and Sterol regulatory element binding proteins (SREBP) (137). LXRs can be activated endogenously by oxysterols and intermediate precursors in the cholesterol biosynthetic pathway. Apart from oxysterols, high affinity synthetic agonists, such as GW3965 and T0901317 (TO) have also been widely used as compounds to study LXR signaling pathways (89).

LXR are well known for their crucial role in cellular cholesterol, lipid and glucose homeostasis (138). Furthermore, studies have identified their potential role in neural development, maintenance of blood brain barrier integrity and water homeostasis (139–141). Moreover, the potential anti-inflammatory role of LXRs by exerting anti-inflammatory effects in various cellular and physiological processes has been demonstrated in a variety of cell types (142). The activation of LXR has been linked with the suppression of transcription of inflammatory mediators such as AP-1 and NFκB. Further studies unravel the implication of LXR activation in the maintenance of endothelial barrier functionality (141,143).

Under conditions of cholesterol overload, cells of the peripheral tissues activate LXR (144). Activated LXR induces the expression of cholesterol transport genes such as ABCA1 and ABCG1, which facilitate cholesterol transfer to lipid-poor apoA1-HDL and mature high density lipoproteins (HDL) respectively (145). Through this mechanism called the reverse cholesterol pathway, excess cholesterol from the peripheral tissues will be transported back to the liver where it will be redistributed to other tissues or removed from the body in the form of

bile acids (146). In conditions of GDM, the increased levels of oxysterols trigger the activation of LXR which induces genes involved in cholesterol homeostasis. Through this protective mechanism, the fetoplacental endothelium function is maintained by LXR even in the conditions of GDM (108). Human placental trophoblasts take up LDL and HDL as they express receptors for LDL receptor and SR-B1 respectively (147,148). For the release of maternal cholesterol from the placenta to fetal circulation, the fpEC at the chorionic plate play a significant role. Those cells efficiently release cholesterol through two major cholesterol transporters ABCA1 and ABCG1, which transport cholesterol to lipid free apoA-1 or ApoE and HDL respectively (149). Phospholipid transfer protein (PLTP) found to be expressed in human fpEC additionally delivers cholesterol from fpEC to fetal HDL (21).

A growing number of studies identify several mechanisms by which LXR suppresses inflammation in cells. One mechanism termed trans-repression proposes that LXR in the monomer state gets SUMOylated by small ubiquitin-related modifier 2/3 (SUMO 2/3) proteins in the presence of LXR agonists. The sumoylated LXR then prevents the removal of repressor complexes such as nuclear receptor co-repressor 1 (NCoR) protein present on the promoter region of pro-inflammatory genes such as NFκB and AP1 and other TLR response genes, thereby hindering their transcription (150). They are also identified in trans-repressing signal transducer and activator of transcription 1 (STAT1) inflammatory signaling by preventing the interaction of STAT1 with the promoters of inflammatory genes such as IRF1, TNF-α and IL-6 (151).

In addition, the ability of LXR to inhibit inflammation in the absence of sumoylation has also been demonstrated. Upon activation, LXR upregulates the expression of cholesterol efflux genes ABCA1 and ABCG1, leading to an increased cholesterol efflux from the cholesterol rich lipid raft domains in the plasma membrane and thereby affecting the membrane lipid organizations (140,152). The altered membrane lipid composition suppresses the activation of TLR. In macrophages, activation of LXR is shown to inhibit signaling from TLRs 2, 4 and 9 to their downstream effectors such as NFκB and MAPK by disrupting the recruitment of MyD88 and TRAF6. ABCA1 is predominantly involved in LXR-mediated anti-inflammatory pathways (140). These findings potentially link cholesterol metabolism to inflammation through ABCA1-dependent regulation.

LXR synthetic agonists have been shown to exhibit anti-inflammatory and anti-atherogenic effects by repressing inflammatory genes in macrophages, which are attributed to their ability to regulate cholesterol metabolism through regulating various target genes such as PLTP, ABCA1 and ABCG1 (153). A recent study demonstrates the ability of TO to increase the level of alternative splice short form of MyD88 mRNA by downregulating the splicing factor 3 subunit 1 (SF3A1) expression causing the dampening of TLR4 inflammatory signaling cascade (154). LXR activation has been implicated in the modulation of the function of various immune cells, including macrophages, dendritic cells, and T cells. Its activation by agonists is associated with decreased production of inflammatory mediators such as cytokines and chemokines in a variety of cell types and chronic inflammatory diseases (155). LXRs also modulate dendritic cell maturation and function, contributing to immunomodulation (156). Additionally, LXR activation can regulate the differentiation of T cells and cytokine production, influencing the overall immune responses (157). Thus, LXR has potential therapeutic implications as targets for metabolic and inflammatory disorders such as atherosclerosis, diabetes, autoimmunity, cancer and neurodegenerative diseases as they regulate diverse pathways in development, reproduction, metabolism, immunity and inflammation. Recent insights into LXR signaling suggest future targeting strategies aiming at increasing LXR in specific cell types (158).

1.5.1 Role of LXR activation on placental function and pregnancy related inflammation

Knowledge on the effects of LXR activation on inflammation during pregnancy pathologies is limited. LXR activation has been investigated for its potential impact on oxysterol-induced inflammatory responses in placental trophoblasts, as evidenced by attenuated oxysterol- and LPS-induced cytokine release in LXR-activated cells (94). LXR has been identified as an important factor in early pregnancy lipogenesis to protect against abnormalities in fetoplacental lipid homeostasis (159). Meanwhile, it is well accepted that an excessive tissue inflammation accompanied by increased production of pro-inflammatory factors and a decreased activity of regulatory immune cells contribute to the development and progression of PE and thereby dysfunction of the placenta (160). Given the extensive knowledge of the regulatory effects of LXR on TLR activation, pharmacological modulation of LXR holds promise as a potential treatment for inflammation in such pregnancy-related conditions. The

functional role of LXR in the placenta remains largely unexplored, with limited characterization. However, the available studies dealing with LXR in the placenta have suggested several roles of this receptor, including its involvement in placentation and trophoblast invasion, inhibition of human chorionic gonadotropin secretion, and regulation of cholesterol transport and lipid biosynthesis (161).

These findings suggest that LXR may play an important role in human placentation and in the transport and metabolism of lipids within the fetoplacental unit. The precise mechanisms and implications of LXR in these processes require further investigation. However, these initial observations emphasize the importance of LXR in various aspects of placental function and the regulation of placental inflammation, particularly in response to oxysterols and maternal lipid concentrations. It is therefore important to investigate the relationship between LXR activation and inflammation in the human placenta in order to understand the mechanisms involved.

2. Objectives of the study

Oxysterol concentrations are expected to be elevated in inflammatory pregnancy disorders such as GDM and PE and the fetoplacental endothelium is likely to be exposed to higher levels of oxysterols in these conditions. Higher oxysterol levels contribute to inflammation and disruption of vascular integrity. Several nuclear receptor activations are often associated with anti-inflammatory responses and protective cellular functions. LXR activation has been implicated in the regulation of inflammation. The essential role of LXR activation on placental endothelial cells has yet to be understood. Understanding the effects of LXR activation in placental endothelial cells could provide valuable insights into its potential therapeutic implications in modulating inflammation-related disorders during pregnancy.

Therefore, the specific aims of my thesis were to:

1. Investigate the impact of 7-ketoC and 7β-OHC on inflammatory responses in fetoplacental endothelial cells
2. Study the pro-inflammatory signaling pathways activated by these oxysterols and investigate the underlying mechanism involved.
3. Determine the effects of oxysterols on the functions of fpEC, particularly the intercellular barrier functionality.
4. Investigate the effect of pharmacological activation of LXR on oxysterol-induced inflammation and to identify the mechanism involved.
5. Investigate the potential of LXR activation in maintaining fpEC barrier integrity.

3. Materials and Methods

3.1 Study population

The Ethics Committee of the Medical University of Graz, Austria, approved the present study (29-319 ex 16/17), and all study participants gave voluntary informed consent. The oral glucose tolerance test (OGTT) was performed at 24 weeks of gestation. Subjects with normal OGTT results were grouped as control subjects, and subjects with GDM were diagnosed according to the World Health Organization/The International Association of Diabetes and Pregnancy Study Group (WHO/IADPSG) criteria (162). Those participants who had other pregnancy complications, such as hypertension, PE, HELLP (hemolysis, elevated liver enzymes and low Platelets) syndrome, or any evidence of maternal or fetal infection were excluded from this study. The clinical characteristics of the subjects are listed in Table 1.

Table 1: Subject characteristics

	Parameters	Control (n=14)	GDM (n=10)	p value
Maternal	Age(y)	31±6	32±4.5	ns
	Height (cm)	164±4	162±9	ns
	Pre-pregnancy Weight (kg)	64±7	89±9	$p<0.05$
	Pre-pregnancy BMI (kg/m^2)	24±2	33±7.5	$p<0.01$
	Weight at birth (kg)	75±7	96±9	$p<0.05$
	BMI at birth (kg/m^2)	27.5±3	36±5.8	$p<0.01$
Fetal	Gestational age (wk)	41±2	41±2	ns
	Placental weight (g)	660±70	673±155	ns
	Birth weight (g)	3200±470	3247±419	ns
	Length	49±2	50±2.5	ns
	Ponderal index (g/cm3)	2.49±0.26	2.52±0.39	ns

Data are represented as mean±SD. ns; non-significant

3.2 Isolation and culture of primary human fetoplacental endothelial cells

Fetoplacental endothelial cells were isolated from arteries of the chorionic plate of human term placentas obtained from normal and GDM pregnancies following previously published protocol (20). Briefly, from the apical surface of the chorionic plate, arterial vessels were dissected after removing the amniotic membrane. Endothelial cells were isolated by collagenase/dispase method by perfusing and digesting arteries with Hank's balanced salt solution for 8 minutes (HBSS) (Gibco by Life Technologies, Thermo Fisher Scientific, MA, USA) containing 0.1 U/ml collagenase, 0.8 U/ml dispase II (Roche, Vienna, Austria), and 10 mg/ml penicillin/streptomycin (Gibco by Life Technologies, Thermo Fisher Scientific, MA, USA). The cell suspension was centrifuged (800 rpm, 5 min) and the cell pellet was re-suspended in endothelial cell growth medium (Promocell, Heidelberg, Germany) containing supplements and 10% fetal calf serum (FCS) (Promocell, Heidelberg, Germany) on 1% gelatin (Sigma Aldrich, Missouri, USA) coated plate. FpEC were subjected to immunocytochemistry staining of specific endothelial markers Von-Willebrand Factor (A0082, Agilent, CA, USA) and CD31 (mon60021, Sanbio BV, Uden, Netherlands) to check for purity. Any contamination with non-enothelial cells was checked by fibroblast markers CD90 (DIA100, Dianova, Hamburg, Germany); and MsX Fibroblasts (CBL271, Merck, Darmstadt, Germany) and Smooth muscle marker Actin smooth muscle (M0851, Agilent, CA, USA) and Desmin (M0760, Agilent, CA, USA). For maintaining the fpEC in culture, they were grown in Promocell MV media with 5% FCS and supplemented with Endothelial Cell Growth Supplement, Epidermal Growth Factor (recombinant human), Heparin and Hydrocortisone (Promocell, Heidelberg, Germany) to support the cell growth and function. The cells were passaged, and up to 12 passages were used for experiments. The volume of absolute ethanol used as vehicle for treatment was equivalent to the volume of compounds in all experiments. All compounds used for cell culture experiments are listed in Appendix Table 3.

3.3 RNA isolation and real-time quantitative PCR (RT-qPCR)

Total RNA from fpEC was extracted using the RNeasy Mini kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Briefly, the fpEC monolayer was washed with PBS, and then 0.7 ml of QIAzol lysis reagent (QIAGEN, Hilden, Germany) was added to lyse the cells. The concentration and the purity of isolated RNA were determined by measuring the 260/280 ratio using the Scandrop 250 (Analytik Jena AG, Jena, Germany). For the

generation of cDNA from mRNA, Luna universal reverse transcriptase PCR kit was used (New England Biolabs GmbH Frankfurt, Germany). Thereafter, real-time quantitative PCR (qPCR) was carried out by using Luna universal qPCR reagent (New England Biolabs GmbH Frankfurt, Germany) in CFX96 or CFX384 real-time PCR cycler (Bio-Rad Laboratories Vienna, Austria). All the primer sequences used in this study are listed in Table 2. The efficiency of all the primers used in this study was verified by plotting standard calibration curves. To normalize the gene expression, the housekeeping gene HPRT1 (Hypoxanthine Phosphoribosyltransferase 1) was used and the results were calculated using the $2^{-\Delta\Delta CT}$ method.

Table 2: Primer sequences used for RT-qPCR

Gene (Human)	Primer	Sequence (5'-3')	Amplicon size (bp)
HPRT-1	Forward	GACCAGTCAACAGGGGACAT	111
	Reverse	CTGCATTGTTTTGCCAGTGT	
IL-6	Forward	CCACACAGACAGCCACTCAC	129
	Reverse	TGCCTCTTTGCTGCTTTCAC	
IL-8	Forward	GACCACACTGCGCCAACAC	101
	Reverse	CTTCTCCACAACCCTCTGCAC	
IL-1α	Forward	ATCAGTACCTCACGGCTGCT	189
	Reverse	TGGGTATCTCAGGCATCTCC	
IL-1β	Forward	TGGCAATGAGGATGACTTGTTC	120
	Reverse	TGGTGGTCGGAGATTCGTAG	
TNF-α	Forward	ACGCTCTTCTGCCTGCTG	126
	Reverse	CTTGTCACTCGGGGTTCG	
VCAM-1	Forward	GGGAAGATGGTCGTGATCCTT	89
	Reverse	TCTGGGGTGGTCTCGATTTTA	
ICAM-1	Forward	ATGCCCAGACATCTGTGTCC	112
	Reverse	GGGGTCTCTATGCCCAACAA	

3.4 SDS-PAGE and western blotting

Protein lysis buffer containing protease and phosphatase inhibitor (Roche Diagnostics Mannheim, Germany) was used to lyse the cells. Vigorous vertexing and sonication were used to isolate proteins from the cells. Protein concentration was quantified by bicinchoninic acid assay (BCA) (Thermo Fisher Scientific, MA, USA) and an equal amount of protein was loaded into the gel. To prepare the loading sample, protein samples were mixed with XT loading dye (Biorad, CA, USA) and reducing agent (Biorad, CA, USA) to attain a final concentration of 1x. The loading samples were boiled at 95°C for 5 minutes to denature the proteins. Subsequently,

the proteins were separated based on their molecular weight using SDS-PAGE (Sodium dodecyl-sulfate polyacrylamide gel electrophoresis) on a 4-12% Bis-Tris Midi Gel (Biorad, CA, USA). After SDS-PAGE, proteins from the gels were transferred onto 0.2 μM nitrocellulose membrane (Trans-Blot Turbo Mini Nitrocellulose Transfer Membrane, BioRad, CA, USA). Further, the membranes were blocked in 5% skimmed milk (Bio-Rad, CA, USA) in TBST (Tris-buffered saline with Tween20) for 1 hour at room temperature to prevent non-specific binding. For protein detection, the membranes were probed with primary antibodies specific for the target proteins, while β-actin or α-tubulin was used as the housekeeping control. The primary antibodies were incubated with the membranes overnight at 4°C. The list of all primary antibodies used is provided in Appendix Table 4. Subsequently, appropriate HRP-conjugated secondary antibodies, goat-anti-rabbit HRP-IgG (1: 5000, Biorad, CA, USA), horse-anti-mouse HRP-IgG (1: 5000, Cell Signaling, MA, USA) were used and incubated for 1 hour at room temperature. To visualize the protein bands, an enhanced chemiluminescence (ECL) development method (BioRad, CA, USA) was used. Chemiluminescent signals were detected, imaged, and quantified using a ChemiDoc system (Bio-Rad, CA, USA) and ImageLab software (version 5.2.1, Bio-Rad, CA, USA), respectively.

3.5 Cytokine Multiplex

The concentration of secreted cytokines in the supernatant was measured using procartaplex multiplex immunoassay kit (Thermo Fischer Scientific, MA, USA). Concentration of IL-6, IL-8, IL1-α and TNF-α were measured in the supernatant after treatment of fpEC with oxysterols and LPS at different time points following the user's manual. The fluorescence intensity of the samples was measured using a BioPlex-200 suspension array system (Biorad, CA, USA). All obtained protein concentrations in the supernatant were normalized to total cell protein concentration determined by applying Bicinchoninic acid (BCA) protein quantification assay.

3.6 Immunofluorescence

The fpEC cultured on Lab-Tek chamber slides (Thermo Fisher Scientific, NY, USA) were fixed with 4% paraformaldehyde (PFA) for 10 minutes for immunofluorescence assay. After a TBST wash, cells were blocked with donkey serum for 1 hour prior to incubation with

primary antibodies overnight at 4°C. Next, a conjugated secondary antibody (Thermo Fischer Scientific, MA, USA) was applied for 30 minutes after 5 minutes wash with TBST. DAPI (4′,6-diamidino-2-phenylindole) was added as a nuclei counter stain to the slides for 20 minutes. Sections rinsed again with TBST were mounted with Vectashield mounting medium (Vector Lab, Inc., CA, USA). Leica DM4000 B microscope (Leica Cambridge Ltd, Cambridge, England) equipped with Leica DFC 320 Video camera (Leica Cambridge Ltd, Cambridge, England) was used to acquire and analyze computerized images of sections and cells.

3.7 Flow cytometry

All flow cytometry experiments were carried out on an LSR Fortessa flow cytometer (BD Biosciences, USA). Data were analyzed using the DIVA (BD Biosciences, NJ, USA) and FlowJo v10.7.2 software (Tree Star, Inc. OR, USA). The cells were detached using accutase (A1110501; Gibco, Thermo Fisher Scientific, MA, USA) and resuspended in 40 µL of PBS (Phosphate buffered saline). Fc-receptors were blocked by Fc-blockers (Biolegend, CA, USA) on ice for 10 minutes before staining with antibodies. The cell suspension was incubated with the appropriate dilution of fluorescein isothiocyanate (FITC) conjugated anti-human CD-54 (ICAM-1) antibody (353107; 1:40, Biolegend, CA, USA) and Allophycocyanin (APC) conjugated anti-human CD106 (VCAM-1) antibody (305809; 1:40, Biolegend, CA, USA) for 45 minutes at 4 °C. This incubation allowed for the binding of the antibodies to their respective targets on the cells. To exclude dead cells from the analysis, a dead cell stain called 7-Amino Actinomycin D (7-AAD) (420403; Biolegend, CA, USA) was used that can selectively bind to DNA in cells with compromised membrane integrity, such as dead cells. To exclude doublets, a plotting method involving the height or width against the area was utilized for forward scatter or side scatter measurements. To aid in the identification of non-specific background signals, appropriate isotype controls were employed. Specifically, FITC Mouse IgG1 k (400107, Biolegend, CA, USA) and APC Mouse IgG1 k (555751, CiteAb, Bath, England) isotype controls were used as the negative control.

3.8 LDH cytotoxicity assay

Cell viability after oxysterol treatment at different concentrations was accessed by the presence of lactate dehydrogenase in the cell culture supernatant. The same was quantified using

CyQUANT™ LDH Cytotoxicity Assay kit (Thermo Fisher Scientific, MA, USA) following the manufacturer's protocol. Approximately, 20,000 cells were seeded in each well of a 96 well plates and grown for 48 hours. The cells were then treated with different concentrations of 7-ketoC and 7β-OHC for 24 hours in triplicates. The supernatant was collected and analyzed for the quantification of LDH using the colorimetric method. The absorbance of the colored product was measured at 490 nm and at 680 nm reference wavelength.

3.9 Live cell imaging

All microscopic live-cell imaging experiments were performed on an Olympus IX73 inverted microscope. The microscope was equipped with a CCD Retiga R1 camera (Q-imaging, AZ, USA) and a UApoN340 40× oil immersion objective (Olympus, Tokyo, Japan). A LedHUB® (Omicron, Vienna, Austria) equipped with 340, 385, 455, 470, and 550 nm LEDs in combination with GFP (GFP-3035D, Semrock, NY, USA) or CFP/YFP/RFP (CFP/YFP/mCherry-3X, Semrock, NY, USA) filter set was used for illumination. Data acquisition and control of the fluorescence microscope were performed using Visiview 4.2.01 (Visitron, Puchheim, Germany).

3.10 Mitochondrial membrane potential measurements

Tetramethylrhodamine methyl ester (TMRM) (Thermo Fischer Scientific, MA, USA) was used to measure the mitochondrial membrane potential. The cell-permeant, cationic, red-orange fluorescent dye was excited with a wavelength of 550 nm LED and emission was collected at 600 nm wavelength using CFP/YFP/RFP filter set. The fpEC grown on 6-well plates were incubated with 20 nM TMRM in an experimental storage buffer containing 2 mM Ca2+, 138 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 10 mM HEPES, 2.6 mM $NaHCO_3$, 0.44 mM KH_2PO_4, amino acid, and vitamin mix, 10 mM glucose, 2 mM L-glutamine, 1% Penicillin/Streptomycin, 1% Fungizone, and the pH of the solution was adjusted to 7.4. During the measurement, cells were perfused using a gravity-based perfusion system (NGFI, Graz, Austria) with physiological buffer containing 2 mM Ca^{2+}, 135 mM NaCl, 1 mM MgCl2, 5 mM KCl, 10 mM HEPES, 10 mM glucose and the pH was adjusted to 7.4. After the baseline recording for 2 minutes, cells were exposed to 1 μM FCCP to fully depolarize the mitochondria to measure the minimum values of the membrane potential of each cell. Background-subtracted

(R_{min}) TMRM fluorescence ratio of mitochondrial (R_{mito}) to nucleus (R_{nuc}) region was used as readout.

3.11 Cytosolic calcium measurements

Cytosolic Ca^{2+} flux measurements were performed using Fura-2 AM (Sigma Aldrich, Missouri, USA), a high affinity cell permeable cytosolic free calcium indicator. The fpEC cultured on 6-well plates were incubated with 3.3 μM Fura-2 AM in experimental storage buffer for 20 minutes. During the measurements, cells were perfused with physiological buffer for 2 minutes to obtain the basal Ca^{2+} levels and then perfused with vehicle control (EtOH), thrombin, 7-ketoC or 7β-OHC dissolved in physiological buffer for another 10 minutes and the real time fluorescence was recorded. Fura 2-AM was excited at the UV wavelength 340 and 380 nm and the emission was collected at 510 nm. The ratio of fluorescence of the dye at 340 nm (F_{340}) to the 380 nm (F_{380}) is directly proportional to the cytosolic calcium concentration. The ΔCa^{2+} was calculated as the difference between the basal state and the maximum concentration of calcium after the addition of the compounds.

3.12 Electric cell-substrate impedance sensing (ECIS)

ECIS® Z-Theta device (Applied Biophysics, NY, USA) was used for the real-time monitoring of intercellular resistance changes of the cellular monolayer. For that fpEC were seeded at a density of 40,000 cells per 200μl/well of 96W20idf PET arrays (Applied Biophysics, NY, USA) pre-coated with 10 mM L-cysteine for 10 minutes followed by 1% gelatin solution for 1 hour (Sigma Aldrich, Missouri, USA) and grown until confluence for 2 days. Baseline resistance was measured for 3 hours prior to the addition of treatments. Each treatment was performed in duplicates and the resistance for each well was recorded every 120 seconds for 24 hours after the compound treatment. For measurements involving agonists and inhibitors, the same was added 3 hours before measuring the baseline measurements for another 3 hours. This was followed by the addition of oxysterols or positive control. Every treatment was executed with two technical replications, and the resistance values were normalized based on measurements taken just prior to the introduction of the treatment.

3.13 Monocyte-endothelial cell adhesion assay

Flow cytometry-based monocyte adhesion to endothelial cells was analyzed using the method previously established by Vincent V. et al. (163). $5x10^5$ fpEC were seeded in each well of six-well plates and grown until confluence for two days followed by treatments with compounds. CFSE (Carboxyfluorescein succinimidyl ester) (565082 BD Biosciences, NJ, USA) stained THP-1 monocytes were added on top of the endothelial monolayer and incubated for 1 hour at 37°C and 5% CO2. After co-incubation, unbound monocytes were removed by three times washing with PBS. Adherent monocytes and fpEC were dissociated from the culture plates using TrypLE cell dissociation reagent (12604013, Thermo Fisher Scientific, MA, USA). The suspension of monocytes and fpEC were resuspended in PBS and flow cytometry-based quantification of cells was carried out using an LSR Fortessa flow cytometer (BD Biosciences, NJ, USA). THP-1 cells, labeled with CFSE, showed positive fluorescence in the FITC channel, whereas fpEC cells did not exhibit any fluorescence. A total of 20,000 events, including both fpEC and THP-1 cells, were acquired. These cells were then plotted on a histogram with CFSE fluorescence on the X-axis and cell count on the Y-axis. A vertical gate was used to distinguish CFSE-positive (FITC+) THP-1 monocytes on the right-hand side from CFSE-negative (FITC-) fpEC on the left-hand side. The mean fluorescence intensity of CFSE correlates to the percentage of monocytes attached to fpEC.

3.14 Atomic Force Microscopy

The elastic modulus of individual fetoplacental endothelial cells was measured in static mode using a Flex Bio atomic force microscope (Flex Bio AFM, Liestal, Switzerland) coupled to an optical microscope (Observer Z1, Carl Zeiss, NY, USA). Spectroscopy was performed in liquid environment with soft cantilevers qp-SCont with a resonance frequency of 11 kHz and a spring constant of 0.01 N/m (NanoAndMore, Wetzlar, Germany). Every cantilever was calibrated before the experiment to obtain the spring constant using the software-integrated Sadder method. In liquid, the deflection sensitivity of each cantilever was measured on the cover glass as background and calculated in the AFM software. The AFM cantilever was positioned above the cell monolayer and areas of 60x60 µm with a resolution of 19x19 points were measured. The force curves were obtained by measuring the cantilever deflection at every vertical z-position of the cantilever as it approached and indented the cell. The cantilever descended toward the cell at a velocity of 10 µm/s and a stop value of 500pN was reached. All

force-distance curves were collected and only curves over the nucleus were analyzed with the licensed SPIP software (Image metrology A/S, version 6.6.4, Lyngby, Denmark) according to the Sneddon model (based on Hertz model) (164).

$$F_{Sneddon} = \frac{2}{\pi}\frac{E_{surface}}{\left(1 - v_{surface}^2\right)}\tan(\alpha)\,(s_0 - s)^2$$

Where F is the loading force, π is the radius of the cone tip, v is the Poisson´s ratio, E is Young´s modulus for the surface, α is the tip half cone angle and s_0-s is the indentation. S_0 is the point of zero indentation, and s_0-s denotes the indentation, used with the assumptions Esurface ≪ Etip,

s0 – s ≪ R_{tip}, then no adhesion and no viscoelasticity.

3.15 Statistical analysis

Experiments were performed in technical triplicates of multiple cell preparations (n, biological replicates). Data are presented as mean ± SEM. Appropriate statistical test: Two-tailed Student's paired t-test to compare the means from the two groups under two different experimental conditions, one-way ANOVA or two-way ANOVA followed by Dunnett's or Tukey's test depending on the number of variables and type of comparison followed by post hoc test were used to analyze significant differences between groups. GraphPad Software (Version 8.3.0, Inc., CA, USA) was used for all statistical analyses. $p<0.05$ was considered statistically significant.

4. Results

4.1 Cytokine mRNA expression exhibits no difference between control and GDM fpEC

The basal level expression of cytokines such as IL-6, IL-8, IL-1α, IL-1β, and TNF-α in control and GDM fpEC were analyzed using qPCR. These cytokines are known to have elevated levels in the serum of patients with pregnancy disorders such as GDM (52,165). Our results showed that only IL-6 and IL-8 were notably expressed in control or GDM fpEC, as indicated by low ΔCt (Ct value of the reference gene - Ct value of the gene of interest) values from qPCR. On the other hand, the expression levels of IL-1β and TNF-α were extremely low, as indicated by very high ΔCt values, while the expression of IL-1α was medium but still relatively low (Figure 5). The cytokine mRNA expression showed no significant differences between control and GDM cells.

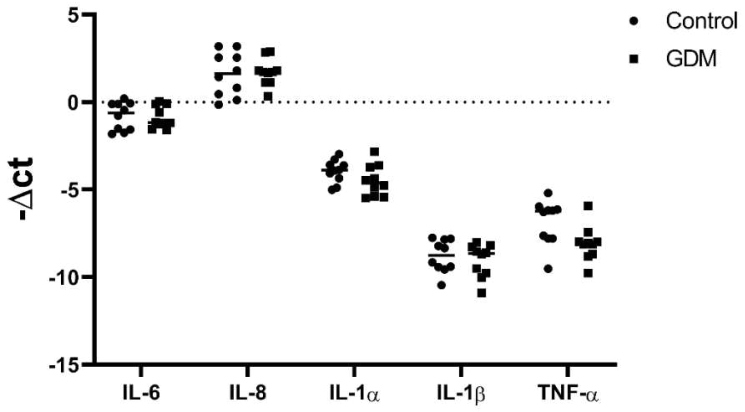


Figure 5: Cytokine mRNA expression exhibits no difference between control and GDM fpEC
The transcription of cytokine mRNA in control and GDM fpEC (n=10) were measured by RT-qPCR using HPRT1 as the housekeeping gene. The Y-axis displays values as negative delta Ct (−ΔCt) to highlight the highly expressed target at the top of the diagram. A higher -ΔCt value indicates greater expression of the target, whereas a low -ΔCt value indicates low expression. IL-6 and IL-8 were observed in higher levels in both control and GDM fpEC, indicated by high -ΔCt value while the other cytokines (IL-1α, IL-1β, and TNF-α) were expressed in low levels at the basal conditions.

4.2 7-KetoC and 7β-OHC exert cytotoxic effects in fpEC at higher concentrations

Several oxysterols are known to induce apoptosis in different cell types at higher concentrations (86,166). Therefore, we analyzed the dose-dependent cytotoxic effects of 7-

ketoC and 7β-OHC in fpEC by measuring the levels of secreted lactate dehydrogenase (LDH) in the cell culture supernatant after 24 hours of stimulation. We used concentrations of 0, 1, 5, 10, 20 and 50 μM for both types of oxysterols. In both control and GDM fpEC, 7-ketoC or 7β-OHC significantly induced apoptosis at 20 μM and 50 μM concentration compared to fpEC treated with vehicle alone (Figure 6). Therefore, we employed a non-toxic concentration of 10 μM of oxysterols for all subsequent studies. Notably, the basal level of apoptotic cells appeared to be significantly higher in GDM compared to normal fpEC (p=0.0049).

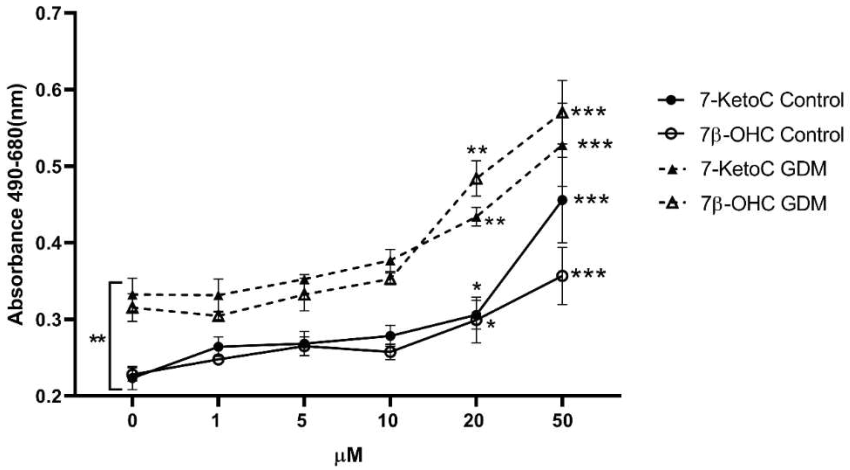


Figure 6: 7-KetoC and 7β-OHC higher than 20 μM exert cytotoxic effects in fpEC

FpEC were seeded on 96 well plates and treated with different concentrations of oxysterols for 24 hours. LDH was measured in the supernatants by measuring the absorbance at the wavelengths 490 nm and 680 nm and the latter served as reference wavelength. Significant differences between the vehicle (0 μM) and treatment groups (different oxysterol concentrations) were calculated using two-way ANOVA followed by Tukey's multiple comparison test. The basal level absorbance of LDH between control and GDM was calculated using two-way ANOVA followed by Sidak's test. *p<0.05, **p<0.01, ***p<0.001. The figure is adapted and modified from George et al. with permission of the publisher (1).

4.3 7-KetoC and 7β-OHC induce pro-inflammatory MAPK and NFκB signaling in fpEC

Oxysterols are endogenous ligands for LXRs but are known to exert inflammation in placental trophoblast cells (94). Moreover, GDM fpEC are pre-disposed to higher concentrations of oxysterols in-utero (108). We investigated whether oxysterols elicit inflammation in control and GDM fpEC at various degrees by assessing the phosphorylation of MAPK family (p38, ERK, JNK) of proteins and p-65 subunit of NFκB. Exposing cells to a

non-toxic concentration of 10 µM oxysterols for a duration of 6 hours notably increased the phosphorylation levels of MAPK proteins and p-65 NFκB in both types of cells, indicating the activation of the TLR4 inflammatory signaling cascade (Figure 8). Interestingly, both control (Figure 7A and B) and GDM fpEC (Figure 7B and D) exhibited similar degrees of phosphorylation in response to oxysterol stimulation.

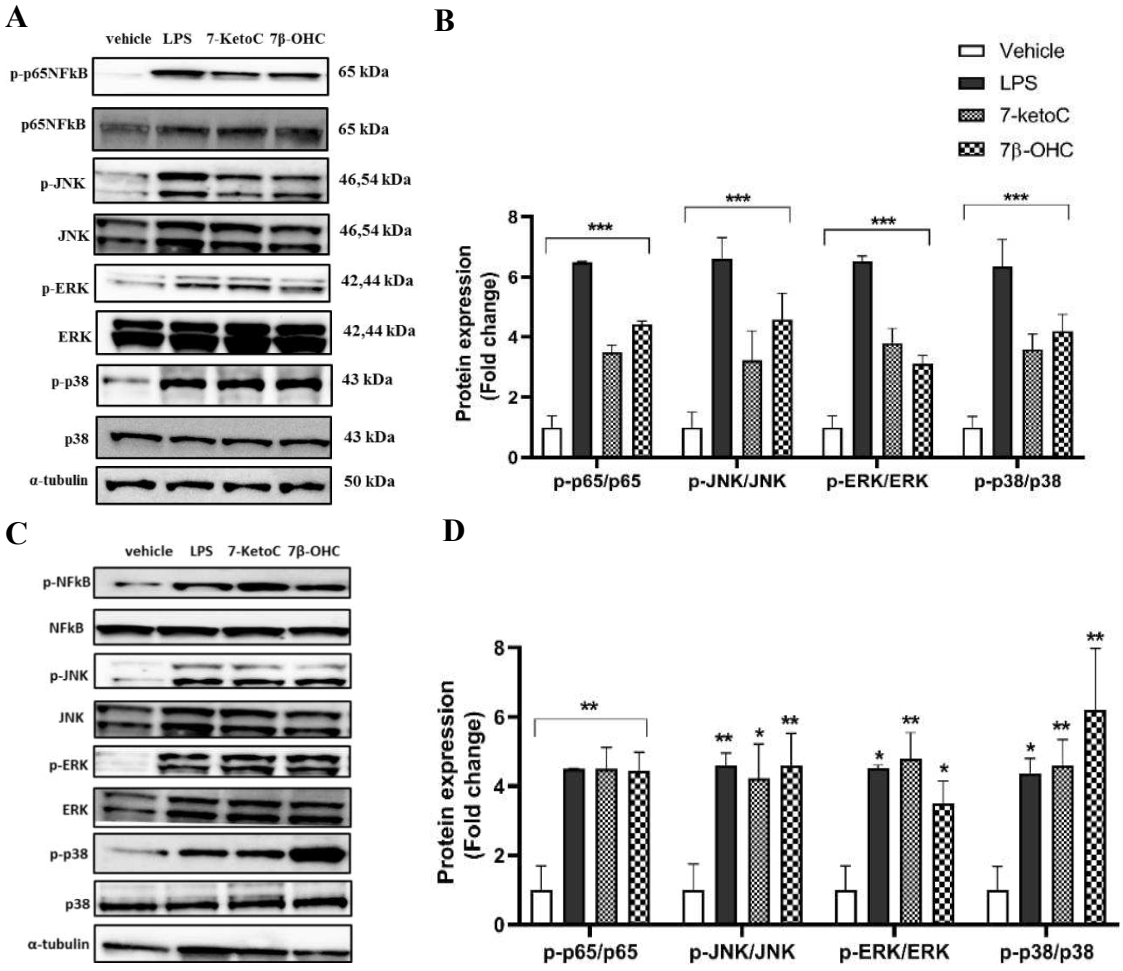


Figure 7: TLR4 signaling is activated in fpEC exposed to oxysterols

Representative western blot shows induction of MAPK and NFκB signaling in **(A)** control and **(C)** GDM fpEC following oxysterol stimulation for 6 hours. LPS (100 ng/µl) served as positive control**.** Densitometric analysis of western blots from **(B)** control and **(D)** GDM fpEC (n=6). Data are presented as mean±SEM. Statistically significant differences between vehicle and treatment groups were calculated using two-way ANOVA, followed by Dunnett's post hoc test. *p<0.05, **p<0.01, and ***p<0.001. The figure is adapted and modified from George et al. with permission of the publisher (1).

4.4 7-KetoC and 7β-OHC induce p-65 NFκB nuclear translocation in fpEC

Upon phosphorylation of the p65 subunit of NFκB, it undergoes translocation to the nucleus, where it acts as a transcription factor and initiates the transcription of inflammatory cytokines such as IL-6, IL-8, TNF-α, and so on (167,168). As oxysterol treatment induced p-65 phosphorylation in fpEC regardless of the disease condition, we aimed to determine if the phosphorylation is followed by translocation to the nucleus in fpEC. To investigate this, we performed immunofluorescence staining of control fpEC to visualize the p-65 nuclear stain. As expected, we observed that 7-ketoC and 7β-OHC increased p65 phosphorylation and its translocation to the nucleus in fpEC compared to the vehicle (Figure 8).

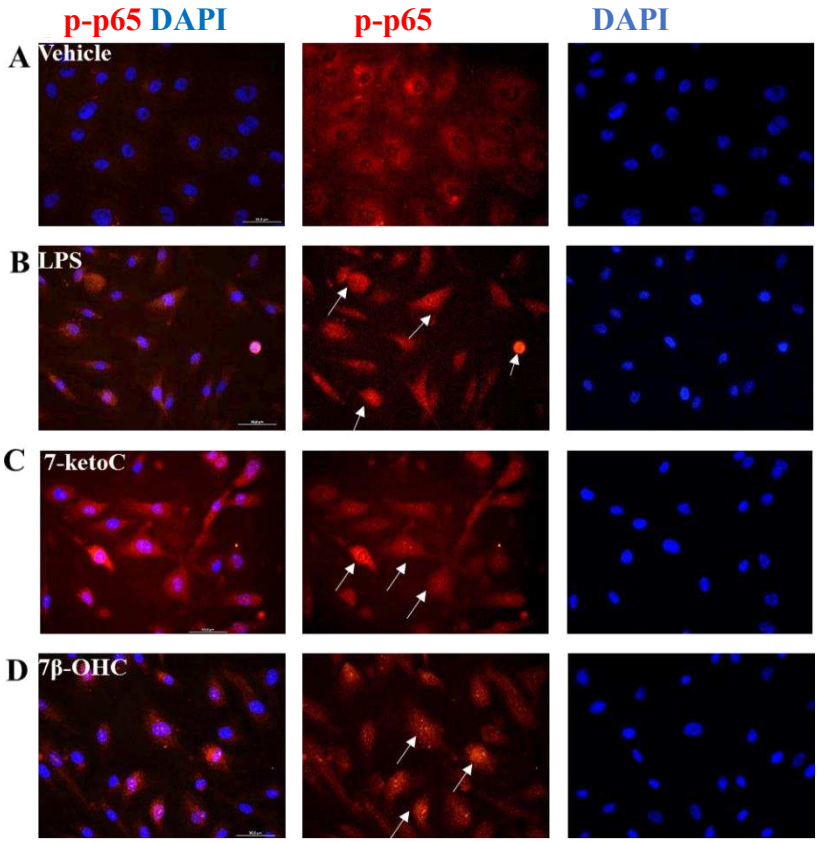


Figure 8: 7-KetoC and 7β-OHC induced p-65 NFκB **nuclear translocation in fpEC**

Exposing fpEC to oxysterol for 6 hours resulted in elevated phosphorylation and the translocation of the phosphorylated p-65 NFκB subunit into the nucleus (n=4). Cells were fixed, permeabilized, and stained for p-p65 (Red) and Nuclei (Blue) with DAPI. The left panel shows the merged images. Scale bar 50 µm. FpEC treated with **(A)** vehicle control exhibited lesser phosphorylation and nuclear staining of p-

p65 compared to **(C)** 7-ketoC or **(D)** 7β-OHC treated cells. White arrows indicate notable phospho-p65 staining in the nucleus. **(B)** 100ng/ml LPS was used as the positive control.

4.5 7-KetoC and 7β-OHC induce transcription but not translation of pro-inflammatory cytokines

To investigate the alterations in cytokine expression in fpEC exposed to oxysterols, we treated control and GDM fpEC with 10 μM of 7-ketoC or 7β-OHC and subsequently quantified the levels of inflammatory cytokines such as IL-6, IL-8, IL-1β, and TNF-α. We excluded IL-1β from further analysis as its basal mRNA expression in fpEC was negligible. Our results demonstrated that treatment with 7-ketoC and 7β-OHC significantly increased the mRNA levels of IL-6 (Figure 9A) and IL-8 (Figure 9B) in both control and GDM fpEC compared to the vehicle. However, both oxysterols significantly induced the transcription of IL-1α (Figure 9C) or TNF-α (Figure 9D) only in GDM fpEC while having no significant effect on control fpEC compared to the vehicle. In contrast, LPS induced significant expression of IL-1α and TNF-α in control fpEC. Subsequently, we collected the cell-culture supernatant at different time intervals (3, 6, 12, 24) hours after exposure to oxysterols to determine the concentration of the secreted cytokines, which serves as an indicator of their translation. Contrary to our expectations, we did not observe any significant changes in the secretion of cytokines from fpEC exposed to oxysterols compared to the vehicle in either control fpEC (Figure 9E, G, I and K) or GDM fpEC (figure 9F, H, J and L). However, treatment with LPS resulted in a significant increase in cytokine secretion in both cell types. Due to the notably low concentration of secreted IL-1α and TNF-α in response to oxysterols or LPS (less than 15 pg/ml), we opted to omit these cytokines from further studies.

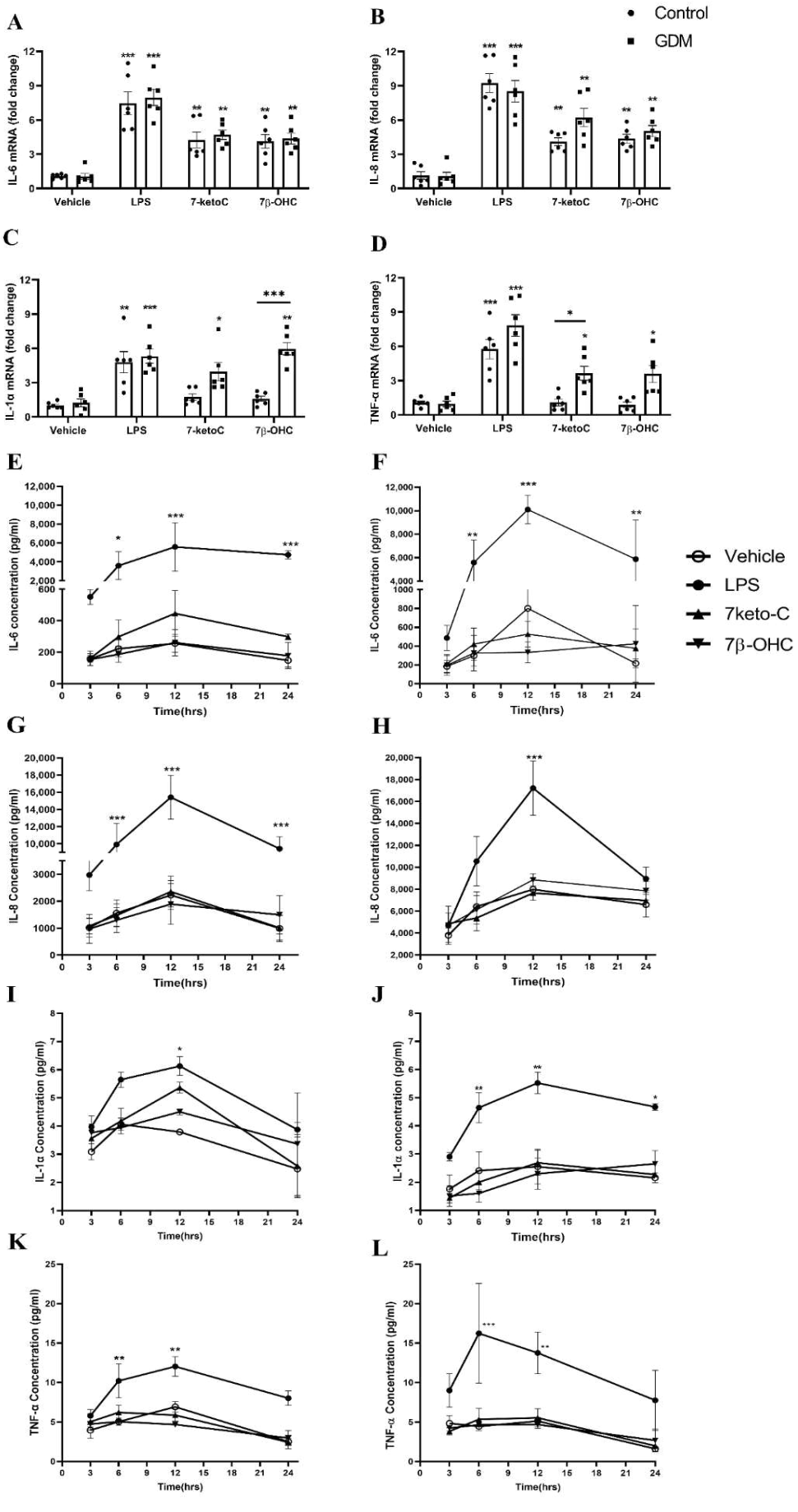

A
B
Control
GDM
IL-6 mRNA (fold change)
IL-8 mRNA (fold change)
Vehicle
LPS
7-ketoC
7β-OHC
C
D
IL-1α mRNA (fold change)
TNF-α mRNA (fold change)
E
F
IL-6 concentration (pg/ml)
IL-6 Concentration (pg/ml)
Time(hrs)
Vehicle
LPS
7keto-C
7β-OHC
G
H
IL-8 Concentration (pg/ml)
I
J
IL-1α Concentration (pg/ml)
IL-1α concentration (pg/ml)
K
L
TNF-α Concentration (pg/ml)

Figure 9: 7-KetoC and 7β-OHC enhance transcription but not translation of cytokines in fpEC **(A)** IL-6 and **(B)** IL-8 mRNA were upregulated by 7-ketoC or 7β-OHC in control and GDM fpEC compared to the vehicle (n=6) following 24 hours of exposure. **(C)** IL-1α and **(D)** TNF-α were significantly induced by oxysterols only in GDM but not in control fpEC (n=6). The expression of IL-1α by 7β-OHC and TNF-α by 7-ketoC in GDM was significantly higher than in control fpEC. Fold changes are relatively expressed to vehicle. LPS (100ng/ul) used as positive control strikingly amplified all cytokine mRNA levels. Significant changes in the secreted levels of IL-6 in **(E)** control and **(F)** GDM, IL-8 in **(G)** control and **(H)** GDM, IL-1α in **(I)** control and **(J)** GDM, TNF-α in **(K)** control and **(L)** GDM were not observed compared to the vehicle following different time points of treatments with 7-ketoC or 7β-OHC (n=4). LPS [100ng/ul] served as the positive control, induced significant cytokine secretion to the supernatant. Data are presented as mean±SEM. Statistically significant differences between vehicle and treatment groups were calculated using one-way ANOVA, followed by Tukey's post hoc test. Statistically significant differences between control and GDM fpEC were calculated using one-way ANOVA, followed by Sidak's test. *$p<0.05$, **$p<0.01$, and ***$p<0.001$. The figure is adapted and modified from George et al. with permission of the publisher (1).

4.6 Oxysterols enhance ICAM-1 and VCAM-1 expression in fpEC

Intercellular adhesion molecules-1 (ICAM-1) and (VCAM1) are essential in mediating inflammatory responses (169). Their expression on the cell surface is augmented in reaction to inflammatory stimuli (170,171). They bind to leukocyte-specific surface receptors during inflammation and initiate immune responses, enabling the transmigration of leukocytes (169,171). We therefore investigated whether 7-ketoC and 7β-OHC have an impact on the expression of ICAM-1 and VCAM-1 in control or GDM fpEC. Notably, in control fpEC, only ICAM-1 mRNA expression was increased by 7-ketoC or 7β-OHC stimulation, while VCAM-1 expression remained unchanged (Figure 10A and B). However, in GDM fpEC, ICAM-1 and VCAM-1 mRNA were significantly induced by oxysterols (Figure 10A and B). The elevated mRNA levels of ICAM-1 in control and both ICAM-1 and VCAM-1 in GDM following oxysterol treatment reflected similar changes in total cellular protein levels (Figure 10C and D). LPS and oxysterols induced ICAM-1 cell surface expression in control (Figure 10E) as well as in GDM fpEC (Figure 10F). Whereas only LPS enhanced VCAM-1 expression in control (Figure 10H) or in GDM fpEC (Figure 10E). Taken together, only ICAM-1 (Figure 10G), not VCAM-1 (Figure 10J) exhibited a significant increase in cell surface localization following 7-ketoC or 7β-OHC treatment in control or GDM fpEC. Whereas LPS significantly induced the

cell-surface expression of both ICAM-1 and VCAM-1. Interestingly, we observed significantly lower basal level cell surface expression of ICAM-1 on GDM cells compared to the control cells (Figure 10G).

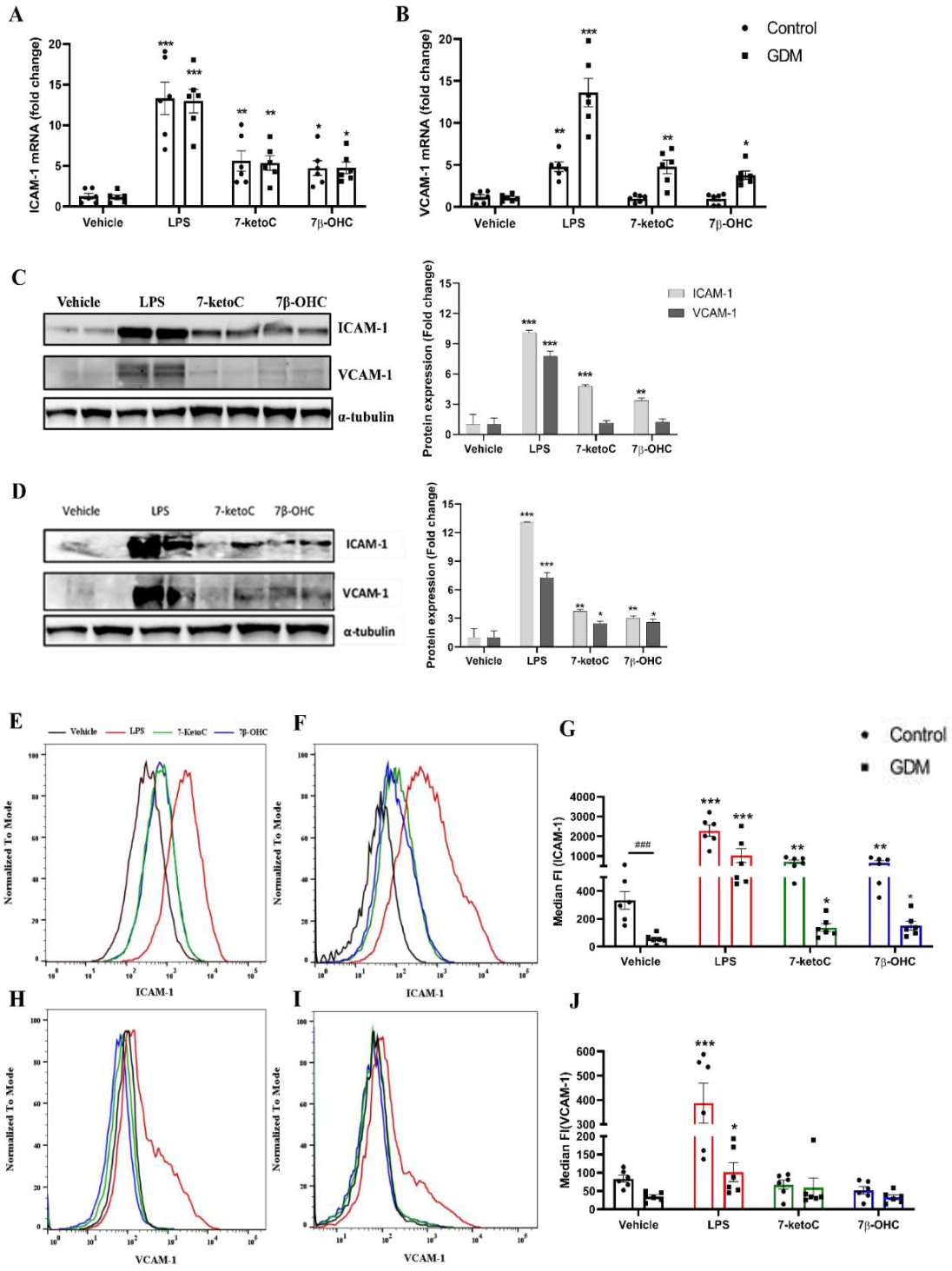


Figure 10: ICAM-1 and VCAM-1 expression is enhanced by oxysterols

Treatment with 7-ketoC or 7β-OHC (10 μm) for 24 hours significantly upregulated **(A)** ICAM-1 mRNA in both control and GDM and **(B)** VCAM-1 mRNA only in GDM fpEC (n=6). LPS (100ng/μl) which served as positive control significantly enhanced both ICAM-1 and VCAM-1 mRNA. **(C)** Representative western blot and bar diagram showing densitometric analysis (n=4) represent induction of only total cellular ICAM-1 protein by oxysterols compared to the vehicle in control fpEC. **(D)** Representative western blot and bar diagram showing densitometric analysis (n=4) represent induction of both ICAM-1 and VCAM-1 total protein following oxysterol treatment in GDM fpEC. Representative FACS histogram showing oxysterols triggered enhanced cell surface expression of ICAM-1 compared to the vehicle in **(E)** control and **(F)** GDM fpEC. **(G)** Bar diagram representing median fluorescence intensity of ICAM-1 in control and GDM fpEC following oxysterol treatment (n=6). FACS histogram depicting enhanced surface expression of VCAM-1 only in cells exposed to LPS in **(H)** control or **(I)** GDM fpEC. **(J)** Bar diagram representing median fluorescence intensity of VCAM-1 in control and GDM fpEC following oxysterol treatment (n=6). Data are presented as mean±SEM. Statistically significant differences between the vehicle and oxysterol treatment group were calculated using one-way ANOVA, followed by Tukey's post hoc test and between control and GDM by Sidak's test (indicated with #). *$p<0.05$, **$p<0.01$, and ***,$^{\#\#\#}p<0.001$. The figure is adapted and modified from George et al. with permission of publisher (1).

4.7 Oxysterols enhance monocyte adhesion to fpEC.

Enhanced ICAM-1 cell surface expression is one of the hallmarks of endothelial activation which facilitates monocyte adhesion to the endothelial cells and promotes extravasation to the subendothelial space (172,173). Therefore, we investigated if enhanced cell surface localization of ICAM-1 triggered by 7-ketoC or 7β-OHC leads to enhancement of THP-1 monocytes adhesion to fpEC. As the effects of oxysterols on GDM and control fpEC were comparable, we focused solely on assessing monocyte adhesion in control cells. As expected, we observed an increase in the attachment of CFSE-tagged THP-1 in fpEC treated with oxysterols for 24 hours, which was indicated by enhanced fluorescence intensity of the fluorophore (Figure 11).

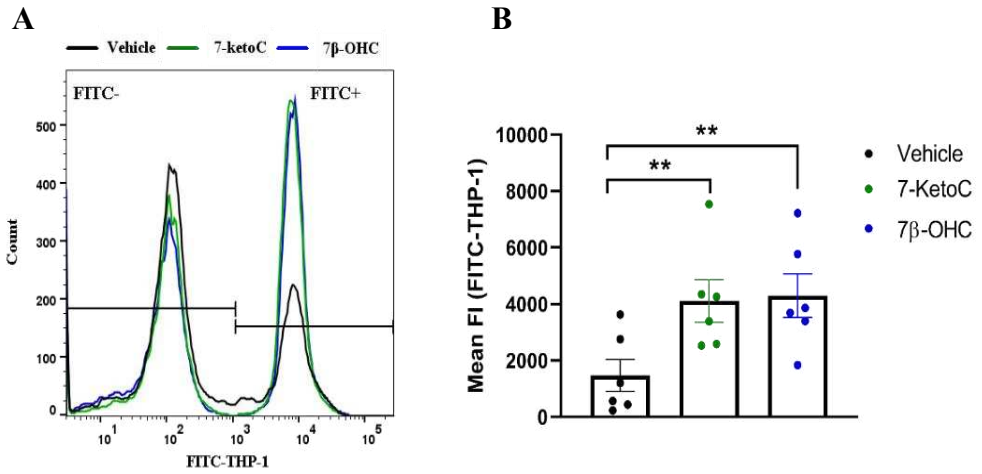


Figure 11: 7-KetoC and 7β-OHC increase monocyte adhesion to fpEC

(A) Representative FACS histogram demonstrates significant increase in the THP-1 fluorescence in cells treated with 7-ketoC or 7β-OHC compared to the vehicle control for 24 hours. **(B)** The mean fluorescence intensity of CFSE labelled THP-1 is significantly higher in the presence of 7-ketoC or 7β-OHC compared to the vehicle (n=6). Data are presented as mean±SEM. Statistically significant differences between different treatments were calculated using one-way ANOVA, followed by Dunnett's post hoc test. **p<0.01.

4.8 Oxysterols induce ROS generation and mitochondrial depolarization

Proper mitochondrial function is essential for overall cell function and cell survival. They are considered as gatekeepers of cell death (174). The mitochondrial membrane potential (Ψm) generated by proton pumps during oxidative phosphorylation across the inner mitochondrial membrane is crucial for ATP production. An increase or decrease in mitochondrial membrane potential can cause cell apoptosis (175). Oxysterols exert cytotoxic effects at mid to high micromolar range (86). As we noticed that oxysterols (7-ketoC and 7β-OHC) at a concentration of 10 µM for 24 hours do not induce cytotoxicity but instead function as pro-inflammatory triggers in fpEC, we extended our investigation to determine whether this concentration of oxysterols impacts the mitochondrial membrane potential (Ψm) of control or GDM fpEC. To test the same, we used TMRM red-orange fluorescent cationic dye which negatively charged mitochondria can sequester in a membrane potential-dependent manner. We observed a decrease in the fluorescence intensity of TMRM, indicating mitochondrial depolarization in cells exposed to 7-ketoC or 7β-OHC in control (Figure 12A) and GDM cells

(Figure 12B) for 24 hours. Following the initial measurement of baseline fluorescence intensity in the cells, FCCP, a compound that disrupts mitochondrial oxidative phosphorylation, was introduced to the cells. This step aimed to completely depolarize the mitochondria and acquire the minimum value for the membrane potential. In the basal state, GDM cells exhibited a slightly higher level of depolarization compared to the control cells, although non-significantly (p=0.10) (Figure 12C). Additionally, we observed that the presence of 10 µM of 7-ketoC or 7β-OHC resulted in an augmentation of ROS generation in control fpEC. This enhancement was evidenced by an increase in the fluorescence intensity of CellRox green reagent, quantified through flow cytometry (Figure 12D). Moreover, previous studies from our lab confirmed the presence of higher levels of ROS in GDM compared to control fpEC (108). It is also established that excessive ROS production can induce the depolarization of the mitochondrial membrane potential (176).

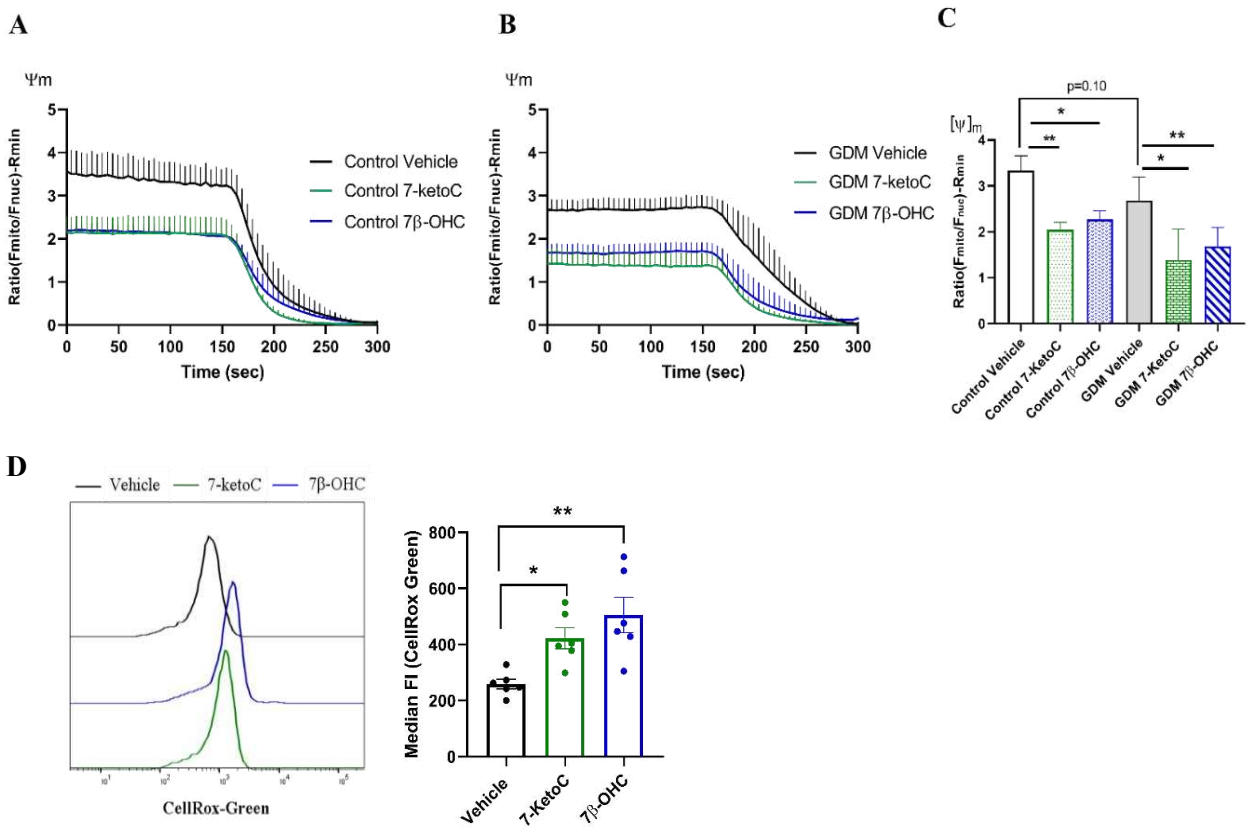


Figure 12: Oxysterols induce ROS generation and mitochondrial depolarization

Graph representing reduction of mitochondrial membrane potential when exposed to 10 µM of 7-ketoC or 7β-OHC for 24 hours compared to vehicle in **(A)** control and **(B)** GDM fpEC (n=5). **(C)** The

histogram represents the ratio of background-subtracted TMRM fluorescence in the mitochondrial (F_{mito}) region to that in the nucleus (F_{nuc}) region, which demonstrates significant reduction in mitochondrial membrane potential in control and GDM cells following 24 hours of exposure to oxysterols. **(D)** 10 μM of 7-ketoC or 7β-OHC treatment in fpEC for 24 hours significantly induced ROS production compared to vehicle in control fpEC. Data are presented as mean±SEM. Statistically significant differences between the groups were calculated using one-way ANOVA, followed by Tukey's post hoc test. *$p<0.05$ and **$p<0.01$

4.9 Oxysterols induce calcium mobilization in fpEC

Calcium (Ca^{2+}) is an essential second messenger in a variety of cell types. Its intracellular concentration is kept low at basal conditions. Any minute change in intracellular Ca^{2+} concentration can trigger a number of diverse signal transduction pathways including cell migration, inflammation, cell contraction, and cell-barrier function (177,178). The increase in Ca^{2+} levels can activate myosin light chain kinase (MLCK), which then phosphorylates the myosin light chain (MLC) in the presence of calcium and calmodulin. This leads to the activation of the actomyosin complex and subsequent cell contraction, which can result in the disruption of cell-cell junctions and contribute to an increase in vascular permeability (179). Furthermore, increased intracellular calcium levels can cause depolarization of mitochondria (180,181). Therefore, we examined the changes in calcium influx (ΔCa^{2+} represents the change in calcium concentration before and after the addition of compounds) in response to oxysterol addition to fpEC. To accomplish this, we subjected control fpEC to a 10-minute perfusion of 7-ketoC or 7β-OHC subsequent to a 2-minute measurement of baseline fluorescence. We observed a significant increase in calcium flux in fpEC when exposed to 7-ketoC or 7β-OHC compared to the vehicle (Figure 13).

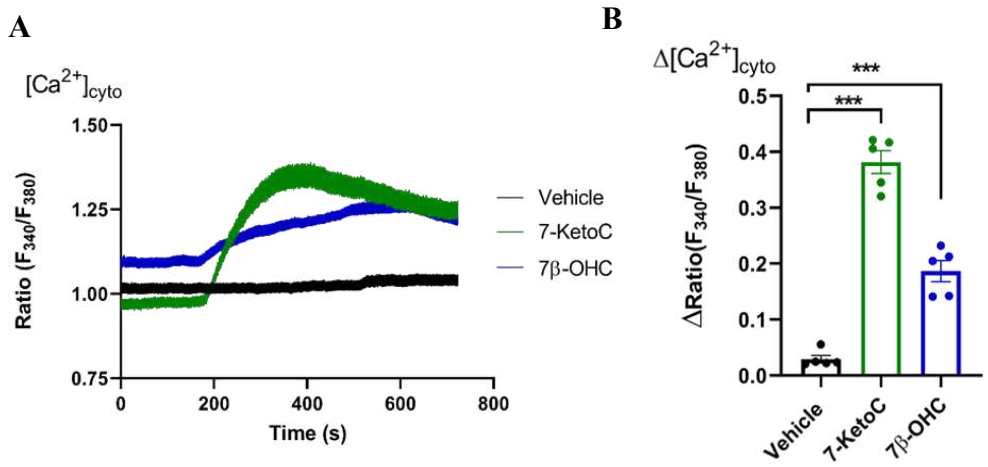


Figure 13: 7-KetoC and 7β-OHC initiate calcium mobilization in fpEC

Baseline calcium fluorescence was observed for 2 minutes, followed by perfusion with vehicle, 7-ketoC or 7β-OHC for 10 minutes and the fluorescence intensity was measured at 340 and 380nm. **(A)** Cells perfused with oxysterols display an increase in calcium mobilization compared to the cells perfused with only vehicle. (**B)** Graph represents changes in calcium concentration (ΔCa^{2+}) obtained by subtracting average baseline fluorescence from the maximum value of concentration (n=5). Data are presented as mean±SEM. Statistically significant differences between different treatments were calculated using one-way ANOVA, followed by Dunnett's post hoc test. ***p<0.001.

4.10 Oxysterols alter the barrier integrity and membrane stiffness of fpEC

The integrity of endothelial cells is largely dependent on cell-cell and cell-matrix junctions. Disruption of these junctions can lead to endothelial dysfunction, which is associated with a variety of cardiovascular diseases. Therefore, maintaining the integrity of endothelial cell junctions is critical for proper cellular functions (182). Endothelial cells undergo remodeling of cell junction and cytoskeleton in response to external stimuli (183). Mechanically stable connections between cells are required for proper endothelial function (184). Vascular endothelial cadherin (VE-cadherin), a component of endothelial adherens junction, is essential for mediating cell-cell junction and plays a key role in the maintenance of vascular integrity (185). Actin filaments help to hold the bundles VE-cadherin adherens junction intact and provide structural stability (184). When exposed to inflammatory or other external stimuli that trigger an increase in cytosolic calcium, actin fibers form contractile stress fibers throughout the cytoplasm, leading to the initiation of acto-myosin contraction. As a result, VE-cadherin

disassembly occurs at the junction, ultimately resulting in vascular permeability (186,187). As we observed increased calcium mobilization by 7-ketoC and 7β-OHC in fpEC, we investigated whether it affects the integrity of the VE-cadherin junction of the cells. FpEC treated with only vehicle appeared with intact VE-cadherin and F-actin localization along the junctions (Figure 14A). Interestingly, we observed that treatment for 1 hour with 7-ketoC (Figure 14B) or 7β-OHC (Figure 14C) induced the formation of stress fibers all over the cytoplasm and led to reduced staining of VE-cadherin at the intercellular junctions compared to vehicle. To further determine the barrier integrity of endothelial monolayer in the presence of oxysterol, we performed ECIS to understand the alterations in trans-endothelial resistance of fpEC monolayer. After adding 7-ketoC or 7β-OHC to the monolayer, we observed a gradual reduction in the resistance. Remarkably, approximately 4 hours post addition, the decrease in electrical resistance achieved statistical significance compared to the vehicle treatment (Figure 14D).

Aberrant formation of stress fibers in the cytoplasm is associated with changes in the mechanistic properties of the cells, such as modifications in plasma membrane rigidity (188). It has been demonstrated that oxysterols can enhance the stiffness of bovine aortic endothelial cells by integrating into the plasma membrane (98). It is well known that alterations in the level of plasma membrane cholesterol have a major influence on the physical properties of the membrane lipid bilayer, such as the ordering of the phospholipids and maintenance of membrane fluidity (189–191). Thus, we conducted experiments using atomic force microscopy to investigate whether oxysterols could influence the membrane stiffness of primary fpEC. Remarkably, we observed a significant increase in membrane stiffness in fpEC treated with 7-ketoC or 7β-OHC for 24 hours compared to the cells treated with vehicle only, as indicated by elevated Young's modulus (Figure 14E).

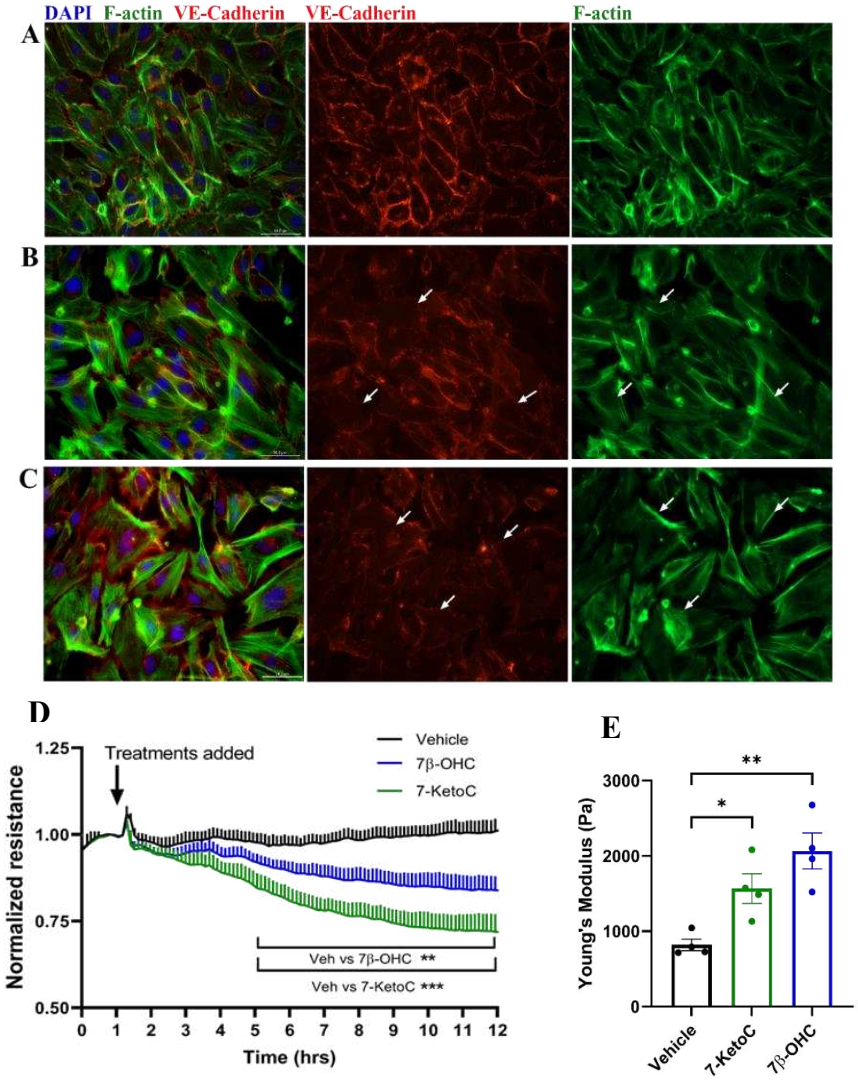


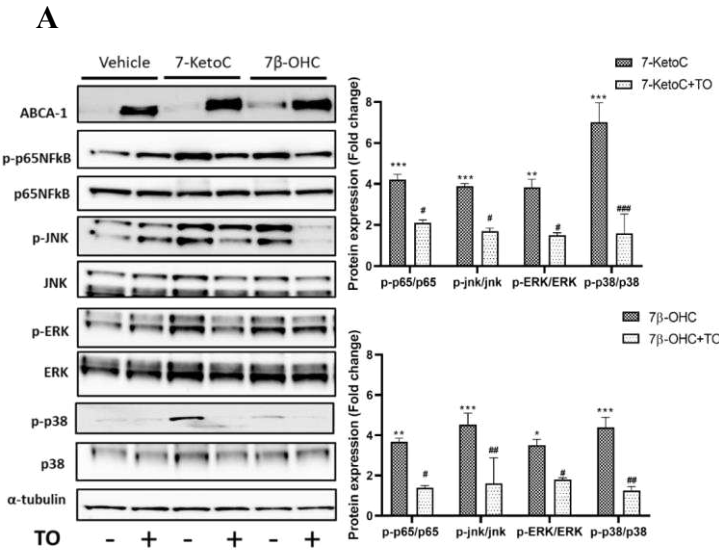
Figure 14: 7-KetoC and 7β-OHC disrupts VE-Cadherin barrier and re-organize actin cytoskeleton

FpEC (n=5) were treated with **(A)** Vehicle **(B)** 7-ketoC **(C)** 7β-OHC and fixed, permeabilized and stained for nuclei (DAPI blue), F-actin (green) and VE-Cadherin (red) and visualized with fluorescent microscopy. Scale bar 50 µm. White arrows indicate disrupted VE-cadherin junction and stress fibers in fpEC exposed to 7-ketoC or 7β-OHC for 1 hour. **(D)** Diagram represents normalized trans-endothelial electrical resistance of confluent monolayer of fpEC exposed to vehicle, 10 µM 7-ketoC, and 7β-OHC. Treatments were added at the time point 1 hour after measuring the baseline resistance. Data presented as mean±SEM. Statistically significant differences between treatments were calculated using two-way ANOVA, followed by Tukey's post hoc test. **p<0.01, and ***p<0.001. **(E)** Young's modulus of fpEC increased after 24 hours of exposure to 7-ketoC or 7β-OHC compared to the vehicle control (n=5), signifying an increase in membrane stiffness. Data presented as mean±SEM. Statistically significant

differences between treatments were calculated using one-way ANOVA, followed by Dunnett's multiple comparison test. **$p<0.01$.

4.11 LXR activation by T0901317 attenuate oxysterol-induced inflammatory signaling in fpEC

Activation of LXR has been linked to the suppression of inflammation (192,193). Studies conducted on animal models have demonstrated that synthetic LXR agonists can alleviate inflammatory disorders (194). Several oxysterols are identified as endogenous ligands for LXR (73). However, 7-ketoC and 7β-OHC treatment resulted in the induction of inflammation in control and GDM fpEC similar to what has been observed in placental trophoblast cells (94). Based on the previous findings, we proposed that the activation of LXR using synthetic agonist T0901317 (TO) may decrease the pro-inflammatory responses induced by oxysterols in fpEC. As the impact of oxysterols on inducing inflammation is not significantly different between GDM and control cells, we continued our studies to identify the underlying mechanisms of induction of inflammation only in control fpEC. To investigate the impact of LXR activation on fpEC, we pre-incubated the cells with 2 μM of TO for 16 hours, followed by the addition of oxysterols (7-ketoC, 7β-OHC). Significant ABCA1 induction confirms LXR activation (Figure 15A). Interestingly, we observed that TO was able to reduce the phosphorylation and hence, the activation of MAPK and p65-NFκB signaling pathways induced by 7-ketoC or 7β-OHC (Figure 15A). TO also attenuated the 7-ketoC or 7β-OHC-induced IL-6, IL-8, and ICAM-1 mRNA expression (Figure 15B and C) and ICAM-1 total protein induction in fpEC (Figure 15D). The vehicle is normalized to one (not indicated in the graph). Furthermore, the activation of LXR was found to reduce ICAM-1 cell surface expression, even when 7-ketoC (Figure 15E) or 7β-OHC (Figure 15F) were present.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7β-OHC
ABCA-1
p-p65NFkB
p65NFkB
p-JNK
JNK
p-ERK
ERK
p-p38
p38
α-tubulin
TO - + - + - +
7-KetoC
7-KetoC+TO
Protein expression (Fold change)
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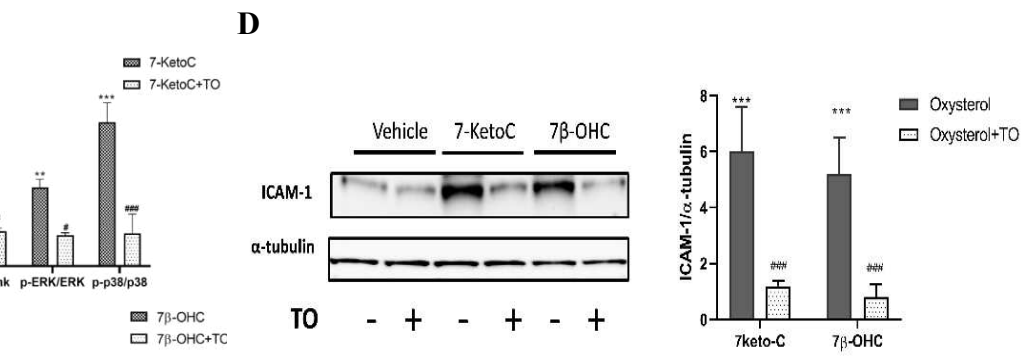
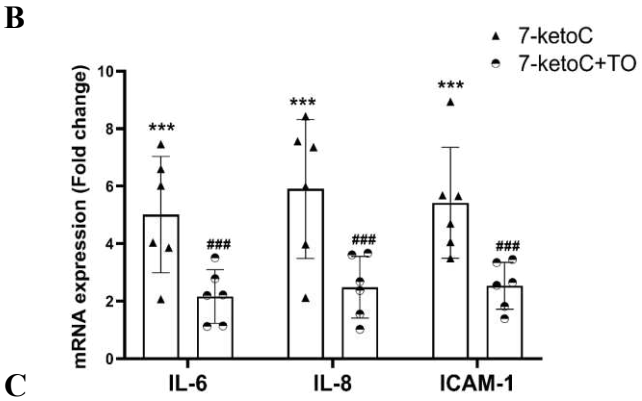
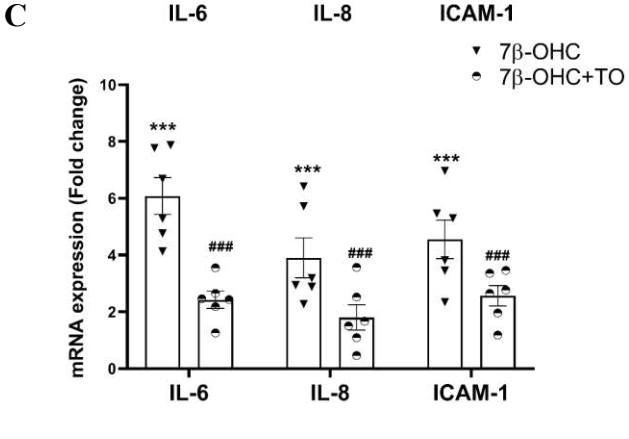
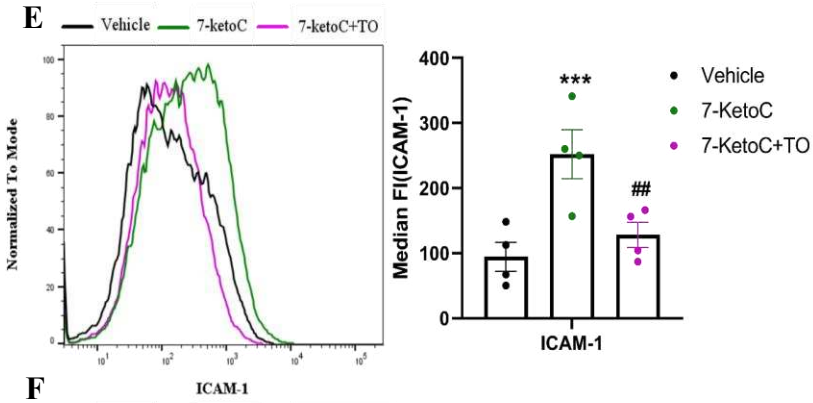
p-jnk/jnk
p-ERK/ERK
p-p38/p38
7β-OHC
7β-OHC+TC


D
Vehicle
7-KetoC
7β-OHC
ICAM-1
α-tubulin
TO - + - + - +
Oxysterol
Oxysterol+TO
ICAM-1/α-tubulin
7keto-C
7β-OHC


B
7-ketoC
7-ketoC+TO
mRNA expression (Fold change)
IL-6
IL-8
ICAM-1


C
7β-OHC
7β-OHC+TO
mRNA expression (Fold change)
IL-6
IL-8
ICAM-1


E
Vehicle
7-ketoC
7-ketoC+TO
Normalized To Mode
ICAM-1
Median FI(ICAM-1)
Vehicle
7-KetoC
7-KetoC+TO

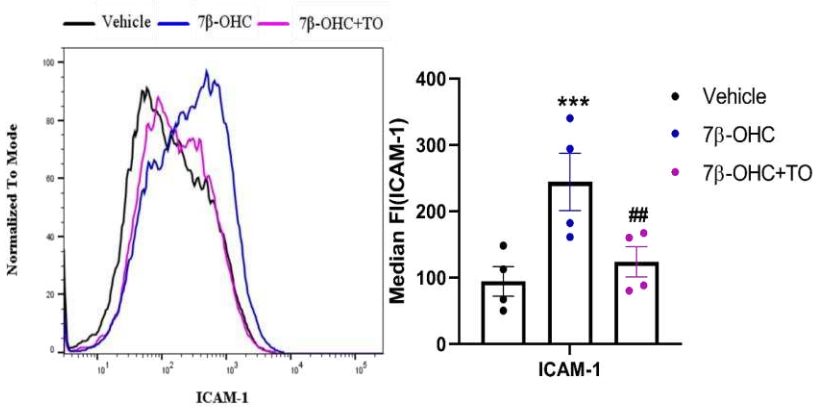

F
Vehicle
7β-OHC
7β-OHC+TO
Normalized To Mode
ICAM-1
Median FI(ICAM-1)
Vehicle
7β-OHC
7β-OHC+TO

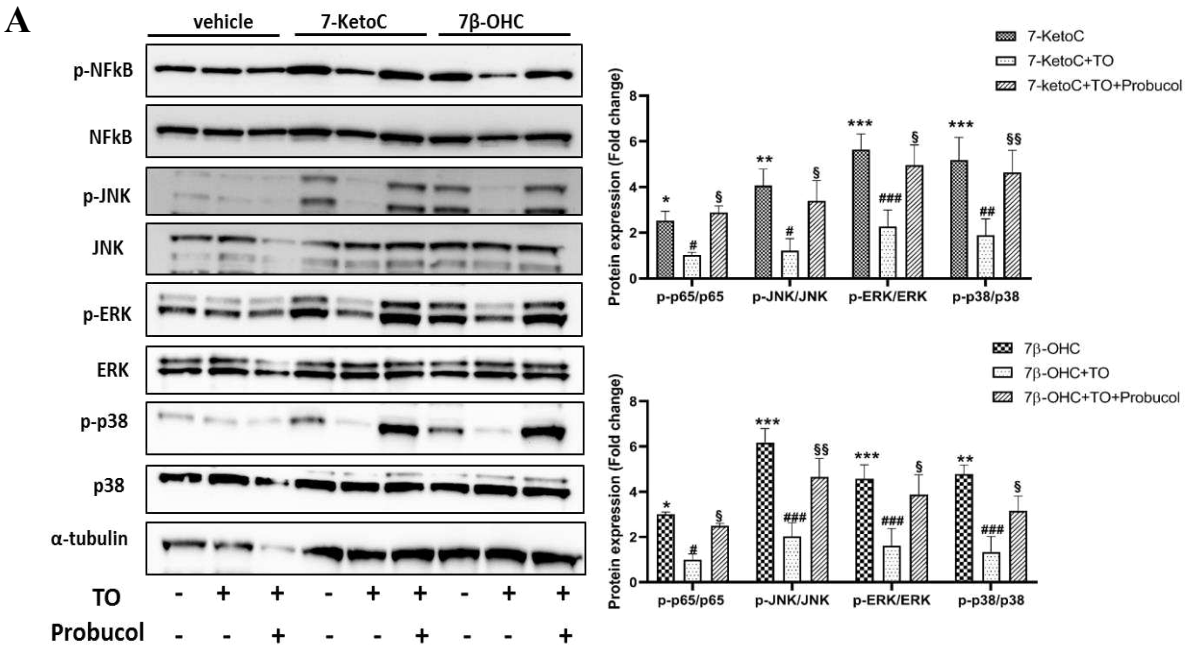
Figure 15: Synthetic LXR agonist T0901317 reduces oxysterol stimulated inflammatory responses in fpEC.

(A) Representative western blot images and corresponding bar diagram depict densitometric analysis revealed notable anti-inflammatory effects of LXR agonist TO in fpEC (n=4) against 7-ketoC or 7β-OHC confirmed by abridged phosphorylation of p-65 NFκB and MAPK (p38, JNK, ERK) proteins. The vehicle was normalized to one (not shown in the graphs), and all fold changes are relative to the vehicle. Reduction in mRNA levels of **(B)** 7-ketoC and **(C)** 7β-OHC-induced IL-6, IL-8 and ICAM-1in LXR-activated cells. **(D)** Representative western blot and densitometric analysis (n=4) demonstrating reduction in oxysterol-induced total ICAM-1 protein expression in fpEC pre-incubated with TO. Representative FACS histogram and respective median fluorescence intensity plot showing reduction in the ICAM-1 cell surface expression levels in LXR-activated cells exposed to **(E)** 7-ketoC and **(F)** 7β-OHC (n=4). Data are presented as mean±SEM. Statistically significant differences between vehicle and oxysterol treatment (indicated with *) and between oxysterol and oxysterol + TO treatment (indicated with #) were calculated using one-way ANOVA, followed by Tukey's post hoc test. $^{\#}$/*$p<0.05$, $^{\#\#}$/**$p<0.01$, and $^{\#\#\#}$/***$p<0.001$. The figure is adapted and modified from George et al. with permission of the publisher (1).

4.12 ABCA1 induction is crucial for LXR-mediated repression of inflammatory signaling in fpEC

Studies have demonstrated that the LXR target gene ABCA1, and not ABCG1, plays a crucial role in the LXR-mediated suppression of inflammatory signaling (140). Reduction in membrane cholesterol content as a result of increased cholesterol efflux from the plasma membrane by ABCA1 dampens TLR activation at the site of the plasma membrane (193). TO-induced expression of ABCA1 protein is approximately 40-fold higher than control whereas, ABCG1 induction is merely 2-fold higher fpEC (108). Therefore, here we hypothesized that ABCA1 is the critical protein involved in the suppression of inflammation by LXR in fpEC. To investigate this, we used an ABCA1 antagonist developed called probucol, which is a diphenolic antilipidemic compound (195). Probucol is an effective inhibitor of ABCA1-mediated cholesterol efflux. In fpEC, probucol diminishes basal and LXR cholesterol release to apoA-1 (149). Here, we examined whether probucol antagonizes ABCA1-mediated suppression of oxysterol-induced inflammatory responses. The fpEC were incubated with 10 μM of probucol along with 2 μM of TO for 16 hours, followed by 6 hours oxysterol treatment for phospho-protein detection and 24 hours for cytokine and ICAM-1 mRNA quantification.

Consistent with expectations, the administration of probucol counteracted the anti-inflammatory effects of TO against oxysterols, as evidenced by the re-induction of phosphorylation of MAPK and NFκB (Figure 16A). (Figure 16A). Probucol's action resulted in the reactivation of these inflammatory signaling pathways associated with inflammation, thereby negating the inhibitory effects of TO and re-induction of cytokine mRNA expression in the presence of 7-ketoC (Figure 16B) or 7β-OHC Figure 16C). Probucol also re-induced total and cell surface ICAM-1 expression in the presence of oxysterols (Figure 16D-F). The results obtained from cells treated with both probucol and oxysterols were comparable to those observed in cells stimulated solely with oxysterols. This indicates that probucol nullified the protective effects of TO and restored the cellular response to a state similar to that induced by oxysterol stimulation alone. We speculate that this effect could stem from the suppression of ABCA1-mediated cholesterol efflux from the plasma membrane, subsequently influencing the activation of TLRs.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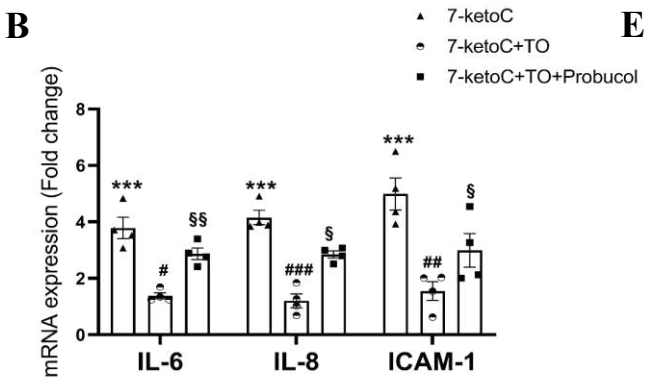
p-JNK
JNK
p-ERK
ERK
p-p38
p38
α-tubulin
TO
Probucol
7-KetoC
7-KetoC+TO
7-ketoC+TO+Probucol
Protein expression (Fold change)
p-p65/p65
p-JNK/JNK
p-ERK/ERK
p-p38/p38
7β-OHC
7β-OHC+TO
7β-OHC+TO+Probucol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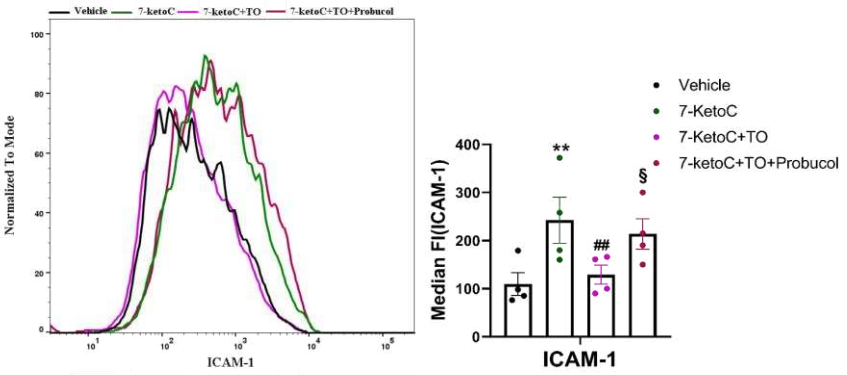
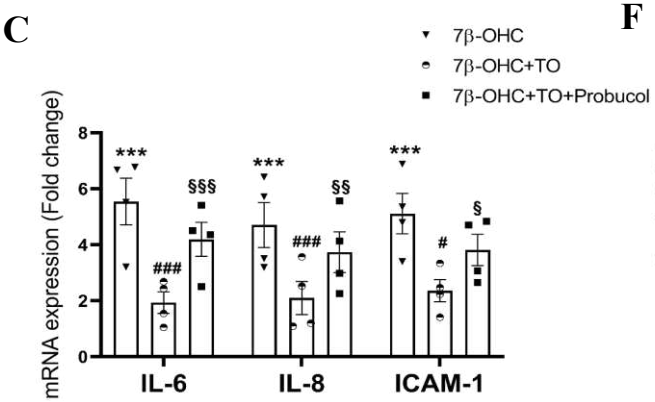
7-ketoC+TO+Probucol
mRNA expression (Fold change)
IL-6
IL-8
ICAM-1


E
Vehicle
7-ketoC
7-ketoC+TO
7-ketoC+TO+Probucol
Normalized To Mode
ICAM-1
Median FI(ICAM-1)


C
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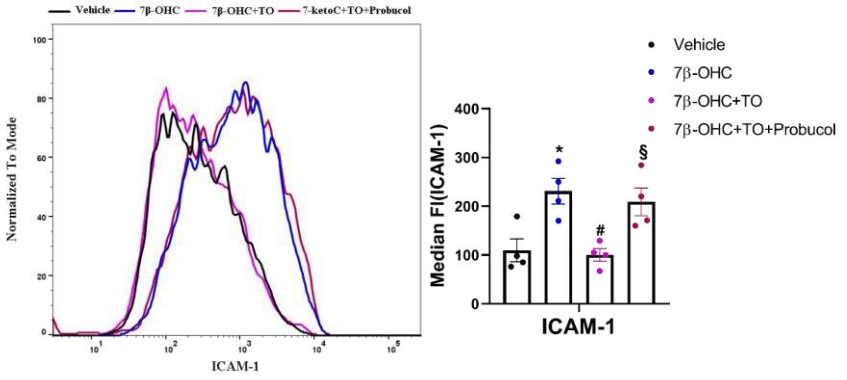
7β-OHC+TO
7β-OHC+TO+Probucol
mRNA expression (Fold change)
IL-6
IL-8
ICAM-1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ICAM-1
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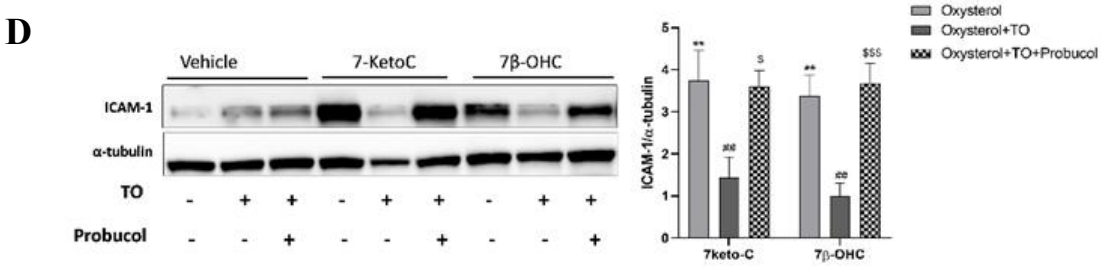
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TO
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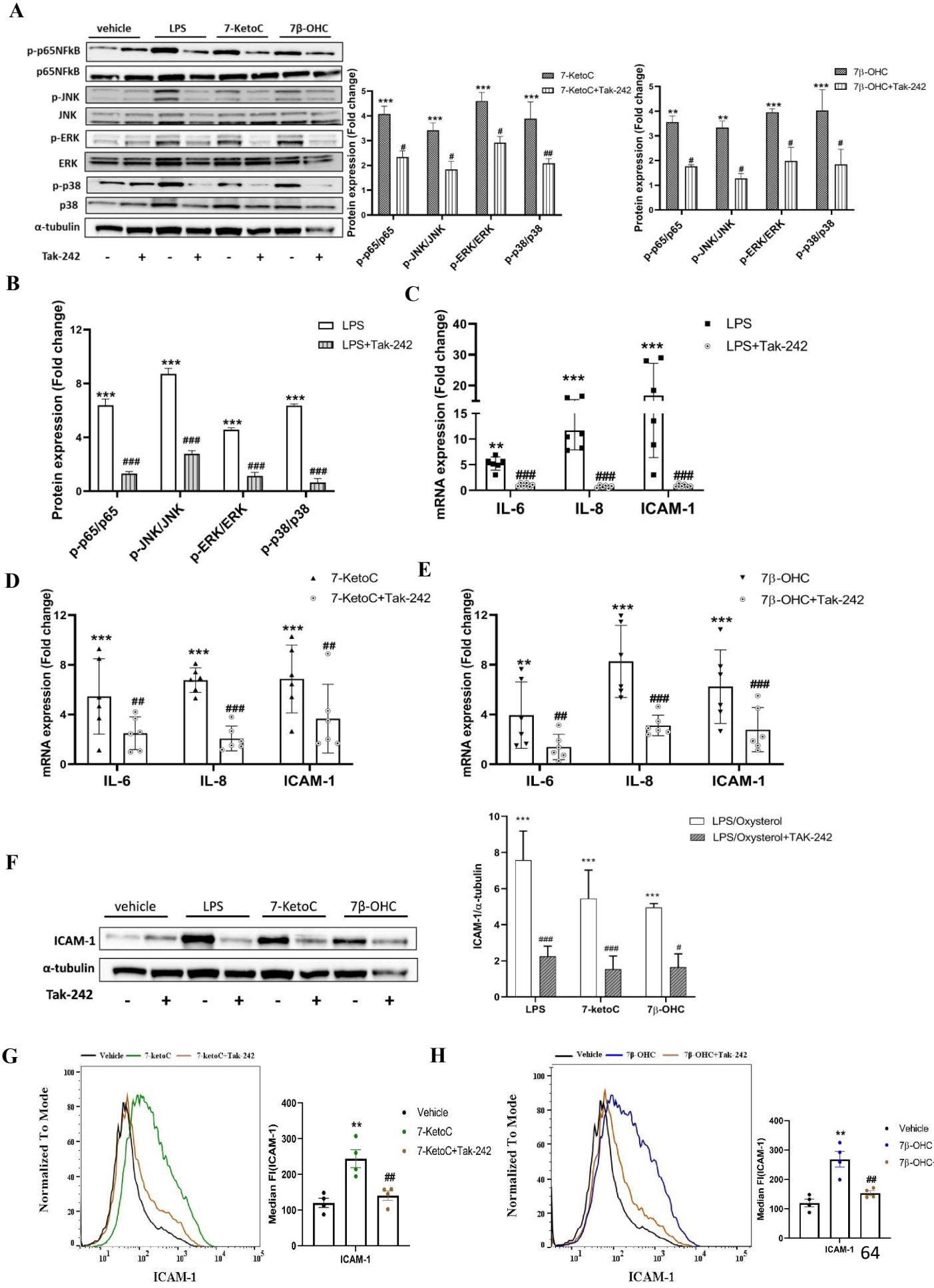
Oxysterol+TO+Probucol
ICAM-1/α-tubulin
7keto-C
7β-OHC

Figure 16: Probucol antagonized the protective effects of T0901317 against oxysterols

(A) Representative Immunoblot and bar diagram showing densitometric analysis (n=4) demonstrating reactivation of MAPK and NFκB signaling in fpEC treated with probucol along with TO followed by 7-ketoC or 7β-OHC similar to what was seen in cells treated with oxysterols alone. The bar diagrams represent the changes in phosphorylation of MAPK and NFκB proteins when exposed to probucol together with TO in the presence of 7-ketoC or 7β-OHC respectively. The vehicle was normalized to one (not shown in the graph) and all treatment groups are relative to the vehicle. IL-6, IL-8 and ICAM-1 mRNA was re-induced in fpEC treated with TO and probucol in the presence of **(B)** 7-ketoC or **(C)** 7β-OHC (n=4). **(D)** Representative western blot and densitometric analysis (n=4) showing the same pattern of total protein expression of ICAM-1 when exposed to probucol. Representative FACS histogram and median fluorescence intensity of cell surface ICAM-1 demonstrating LXR antagonizing effect of probucol in cells treated with **(E)** 7-ketoC or **(F)** 7β-OHC following pre-incubation with TO. Data are presented as mean±SEM. Statistically significant differences between vehicle and oxysterol treatment (indicated with *), between oxysterol and oxysterol + TO treatment (indicated with #) and between oxysterol + TO and oxysterol + TO + probucol were calculated using one-way ANOVA, followed by Tukey's post hoc test. §/ $^{\#}$/*p<0.05, §§/$^{\#\#}$/ **p<0.01, and §§§/$^{\#\#\#}$/***p<0.001. The figure is adapted and modified from George et al. with permission of the publisher (1).

4.13 Oxysterols exert inflammatory responses in fpEC via TLR4-dependent mechanisms

The activation of TLR4 initiates MAPK and NFκB signaling cascades (196). Therefore, we aimed to determine if the inflammatory responses triggered by oxysterol exposure are reliant on TLR4 activation. We pre-incubated fpEC with Tak-242 (TLR4 selective inhibitor) for 2 hours prior to exposure to oxysterols. Interestingly, the phosphorylation of MAPK proteins and p65-NFκB, induced by oxysterols, showed a notable reduction in the presence of Tak-242 (Figure 17A). Additionally, the cytokine mRNA levels and the induction of ICAM-1 (mRNA, total protein, and cell surface expression) triggered by oxysterols were restrained by Tak-242 (Figure 17D-H). Ultrapure LPS which binds to only TLR4, served as positive control and the inflammatory effects of LPS were completely abolished with Tak-242 (Figure 17B-C and I). Our result further gives evidence of TLR4-mediated inflammation induction by 7-ketoC and 7β-OHC.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JNK
p-ERK
ERK
p-p38
p38
α-tubulin
Tak-242
Protein expression (Fold change)
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7-KetoC+Tak-242
7β-OHC
7β-OHC+Tak-242
p-p65/p65
p-JNK/JNK
p-ERK/ERK
p-p38/p38
B
LPS
LPS+Tak-242
C
mRNA expression (Fold change)
IL-6
IL-8
ICAM-1
D
E
F
ICAM-1
LPS/Oxysterol
LPS/Oxysterol+TAK-242
ICAM-1/α-tubulin
G
H
Vehicle
Normalized To Mode
Median FI(ICAM-1)

I

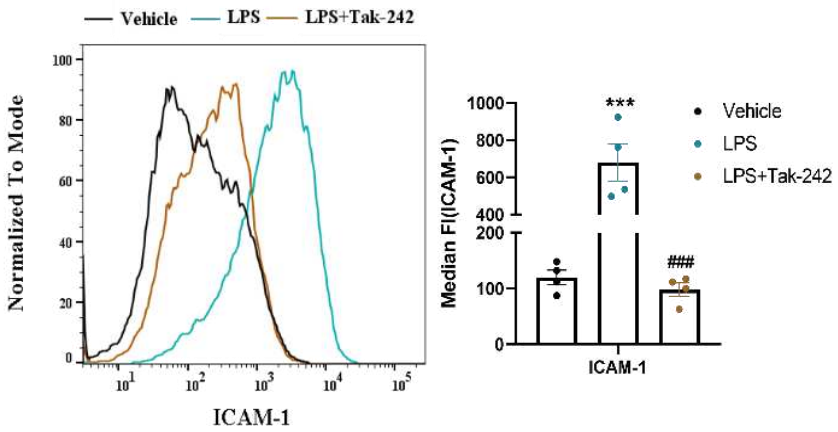

Figure 17: Tak-242 inhibits oxysterol-induced inflammatory responses in fpEC

(A) Representative western blot and bar diagram represent densitometric analysis show a reduction in the activation of p65-NFκB, MAPK-proteins, and expression of ICAM-1 in fpEC exposed to Tak-242 together with 7-ketoC or 7β-OHC compared to cells exposed to oxysterols alone (n=4). All treatment groups are relative to the vehicle which was normalized to one (Vehicle bar is not shown in the graphs) **(B)** Phosphorylation of MAPK and NFκB proteins by LPS was eliminated by Tak-242. **(C)** Similarly, cytokine and ICAM-1 mRNA induction by LPS was completely abolished by TLR inhibition. TLR4 inhibition attenuates IL-6, IL-8 and ICAM-1 mRNA expression induced by **(D)** 7-ketoC and **(E)** 7β-OHC (n=6). **(F)** Representative western blot and the densitometric analysis showing attenuation of 7-ketoC or 7β-OHC induced total cellular ICAM-1 protein in the presence of Tak-242 (n=4). Representative FACS histogram and median fluorescence intensity plot demonstrating attenuation of **(G)** 7-ketoC- or **(H)** 7β-OHC-induced ICAM-1 cell surface expression by Tak-242 (n=4). **(I)** Representative FACS histogram and median fluorescence intensity plot (n=4) demonstrating complete abolition of LPS-induced ICAM-1 cell surface expression by Tak-242. Data are presented as mean±SEM. Statistically significant differences between vehicle and oxysterol treatment (indicated with *) and between oxysterol and oxysterol + Tak-242 treatment (indicated with #) were calculated using two-way ANOVA, followed by Tukey's multiple comparison test. #/*$p<0.05$, ##/ **$p<0.01$, and ###/***$p<0.001$. The figure is adapted and modified from George et al. with permission of publisher (1).

4.14 LXR activation stabilizes VE-Cadherin junction and barrier integrity of fpEC

In addition to its potential anti-inflammatory effects, studies have identified that LXR plays a significant role in maintaining the integrity of the blood-brain barrier in vitro (141). Further, the integrity of the human umbilical vein endothelial cell barrier is enhanced by the activation of LXR (197). Currently, there is limited knowledge regarding the relationship

between LXR activation and the function and integrity of the fetoplacental endothelial barrier. Thus, our study aimed to explore the impact of LXR activation through TO on the endothelial barrier integrity disrupted by oxysterols. For that, the cells were pre-incubated with TO prior to the stimulation with oxysterols. To elucidate the role of LXR in barrier functions, we used LXR antagonist GSK2033 (GSK) to block the activation of LXR. Interestingly, the VE-cadherin arrangement at the cell junctions and the F-actin organization appeared to be more intact in LXR-activated cells (Figure 18B) compared to the vehicle (Figure 18A). The addition of GSK along with TO disrupted the VE-cadherin junction (Figure 18C), which indicates that LXR is essential for the maintenance of cell junction integrity of fpEC. Pre-treatment with TO for 1 hour prior to the addition of 7-ketoC or 7β-OHC resulted in attenuation of adherens junction disruption compared to cells treated solely with oxysterols (Figure 18D, E, G, H). Furthermore, our observations demonstrated that the protective effects of TO against oxysterols were counteracted by the LXR antagonist GSK, leading to the disassembly of VE-cadherin and the formation of actin stress fibers (Figure 18F and I). The trans-endothelial resistance of the monolayer was measured using ECIS assay, which revealed improved integrity of the cell junction barrier of the cells treated with TO. In contrast, the addition of GSK to LXR-activated cells significantly reduced the resistance of the monolayer of fpEC (Figure 18J). Furthermore, fpEC pre-incubated with TO displayed significantly enhanced endothelial barrier resistance in the presence of 7-ketoC or 7β-OHC. However, the addition of GSK along with TO effectively abolished the protective effects of LXR against barrier disruption induced by 7-ketoC or 7β-OHC (Figure 18K and L).

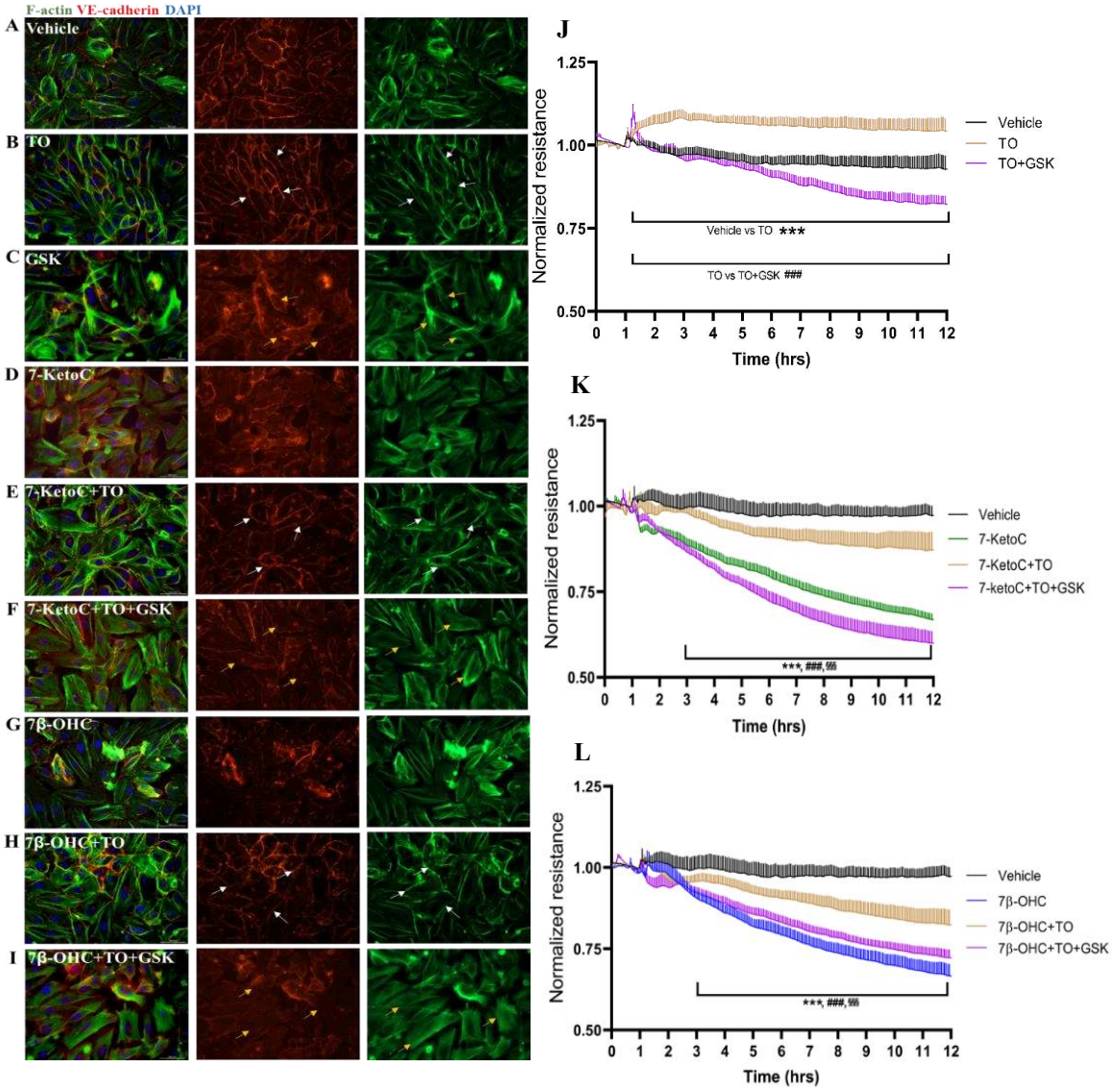

Figure 18: Activation of LXR restored VE-Cadherin junction and barrier integrity in oxysterol stimulated cells

Representative immunofluorescence image of fpEC treated with **(A)** vehicle control showed a well-organized VE-cadherin junction and F-actin organization. **(B)** FpEC treated with TO for 16 hours displayed improvement in the organization of VE-cadherin and F-actin at the cell junction. **(C)** The addition of LXR antagonist GSK, along with TO antagonized the protective effects of TO and induced the formation of stress fibers. As demonstrated previously, fpEC displayed loss of VE-cadherin staining from the cell junction and formation of actin stress fibers after the addition of **(D)** 7-ketoC or **(G)** 7β-OHC. FpEC pre-treated with TO for 16 hours and then exposed to **(E)** 7-ketoC or **(H)** 7β-OHC were able to resist the barrier disruption. When LXR antagonist GSK was added along with TO, the effect of

TO was nullified and the cells returned to a disrupted intercellular VE-cadherin barrier state in the presence of **(F)** 7-ketoC and **(I)** 7β-OHC. White arrows indicate intact VE-cadherin junction and F-actin organization in LXR-activated cells and yellow arrows indicate dissociated VE-cadherin junction and actin stress fibers in the cytoplasm in LXR-deactivated cells by GSK. **(J)** The resistance of the monolayer of fpEC treated with TO for 16 hours was significantly higher compared to the untreated cells (indicated by *). The addition of GSK to the cells pre-treated with TO resulted in a significant reduction in resistance (indicated by #). The reduction in trans-endothelial electrical resistance of fpEC upon the addition of **(K)** 7-ketoC or **(L)** 7β-OHC was significantly reversed upon LXR activation by TO (indicated by #), and it fell back to the oxysterols level when treated with LXR antagonist GSK (indicated by §). All stainings were done with 4 biological replicates. Statistically significant differences between different treatments were calculated using two-way ANOVA, followed by Tukey's post hoc. $^{**,\#\#,\S\S}p<0.01$, and $^{***,\#\#\#,\S\S\S}p<0.001$.

4.15 LXR activation ameliorates oxysterol-induced oxidative stress but failed to significantly maintain mitochondrial bioenergetics disrupted by oxysterols

Several studies have shown that LXR is able to ameliorate oxidative stress by inducing the expression of antioxidant genes (198–200). Explored to a limited extent, some studies have explored LXR's ability to regulate mitochondrial dynamics (201,202). In light of this, we investigated whether LXR could provide protection against the oxidative stress induced by oxysterols. We also sought to ascertain whether LXR could exert control over perturbed mitochondrial bioenergetics and calcium flux within fpEC in the presence of oxysterols. Our findings revealed that pre-incubation with TO leads to a reduction in ROS production triggered by 7-ketoC or 7β-OHC in fpEC. This protective effect was mediated by LXR, as it was negated in the presence of GSK (Figure 19A and B). Surprisingly, the mitochondria remained depolarized in the presence of oxysterols despite pre-incubation with TO, which was contrary to what we expected (Figure 19D). Furthermore, although there was an observable trend in LXR-activated cells, it is worth noting that the reduction of elevated intracellular Ca^{2+} levels induced by 7-ketoC (7-ketoC vs. 7-ketoC+TO p= 0.10) or 7β-OHC (7β-OHC vs. 7β-OHC+TO p=0.08) in fpEC was not statistically significant when treated with TO (Figure 19C). However, TO significantly decreased thrombin-induced ΔCa^{2+} in fpEC (Figure 19E and F).

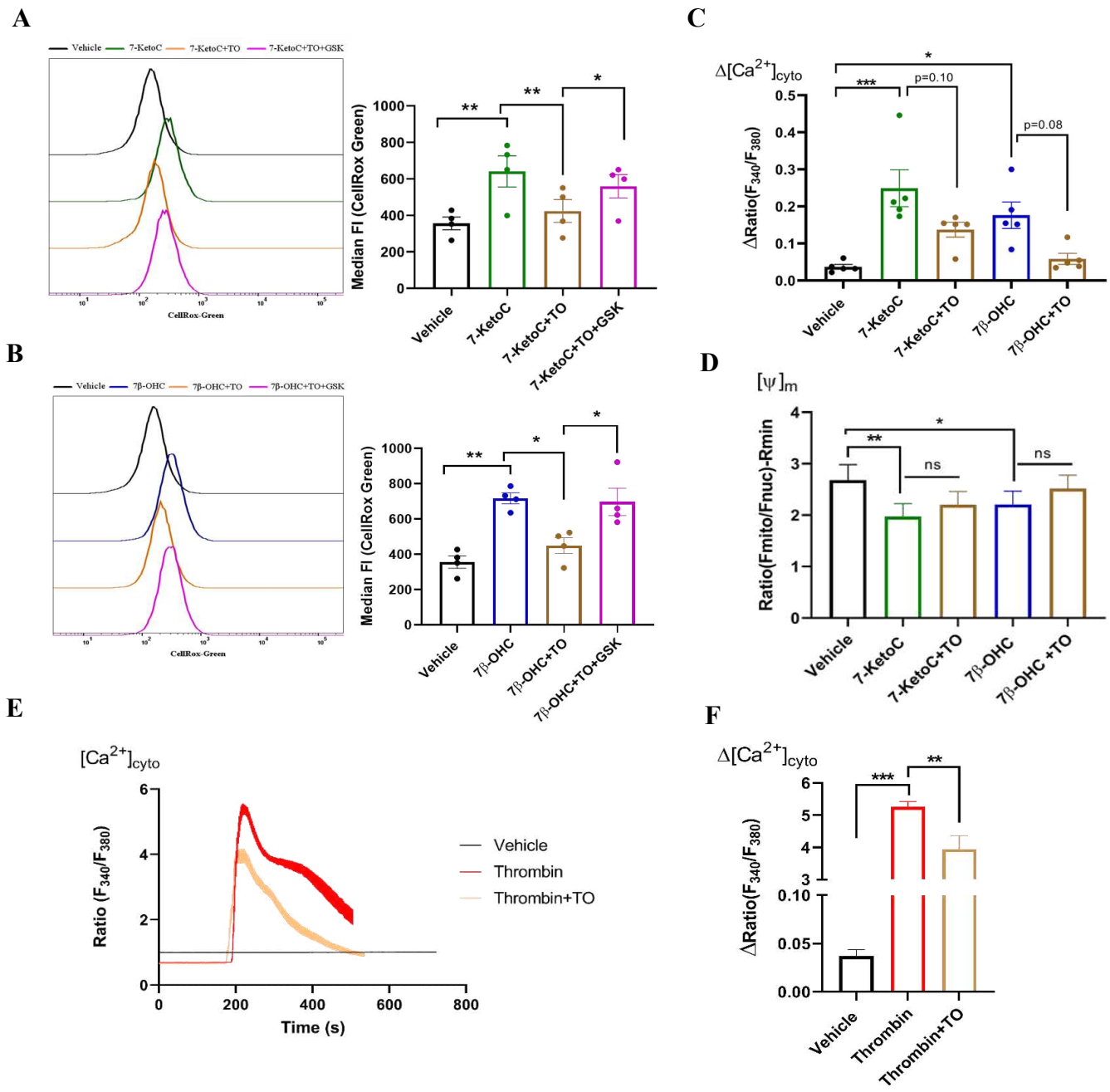

Figure 19: Impact of LXR activation on oxysterol-induced ROS generation, mitochondrial depolarization and calcium flux

Pre-incubation with TO resisted **(A)** 7-ketoC or **(B)** 7β-OHC induced ROS production, and LXR inhibition by GSK abolished this effect in fpEC (n=4). **(C)** LXR activation displayed no positive effect on the mitochondrial function in fpEC in the presence of oxysterols (n=4). **(D)** TO failed to significantly resist the calcium mobilization initiated by oxysterols (n=5). **(E)** Real-time calcium fluorescence showing reduction of thrombin-induced maximum calcium release in LXR-activated cells. **(F)** Graph showing ΔCa^{2+} of thrombin alone and cells pre-incubated with TO followed by thrombin exposure

(n=3). Data are presented as mean±SEM. Statistically significant differences between treatments were calculated using one-way ANOVA followed by Tukey's post hoc test. *p<0.05, **p<0.01, and ***p<0.001.

4.16 Oxysterol-induced monocyte adhesion is lessened in LXR-activated cells

Studies have shown ICAM-1 downregulation in LXR-activated (203). Less cell-adhesion molecule expression on the cell surface hampers monocyte adhesion and subsequent extravasation (204). Our findings align with prior research, as we observed a notable reduction in THP-1 monocyte attachment to fpEC, which were pre-treated with TO and subsequently exposed to oxysterols compared to those treated with oxysterol alone (Figure 20A and B). Intriguingly, the addition of GSK alongside TO reversed this effect, leading to monocyte adhesion levels akin to those observed with oxysterols. This result provides further substantiation for the anti-inflammatory effects of LXR.

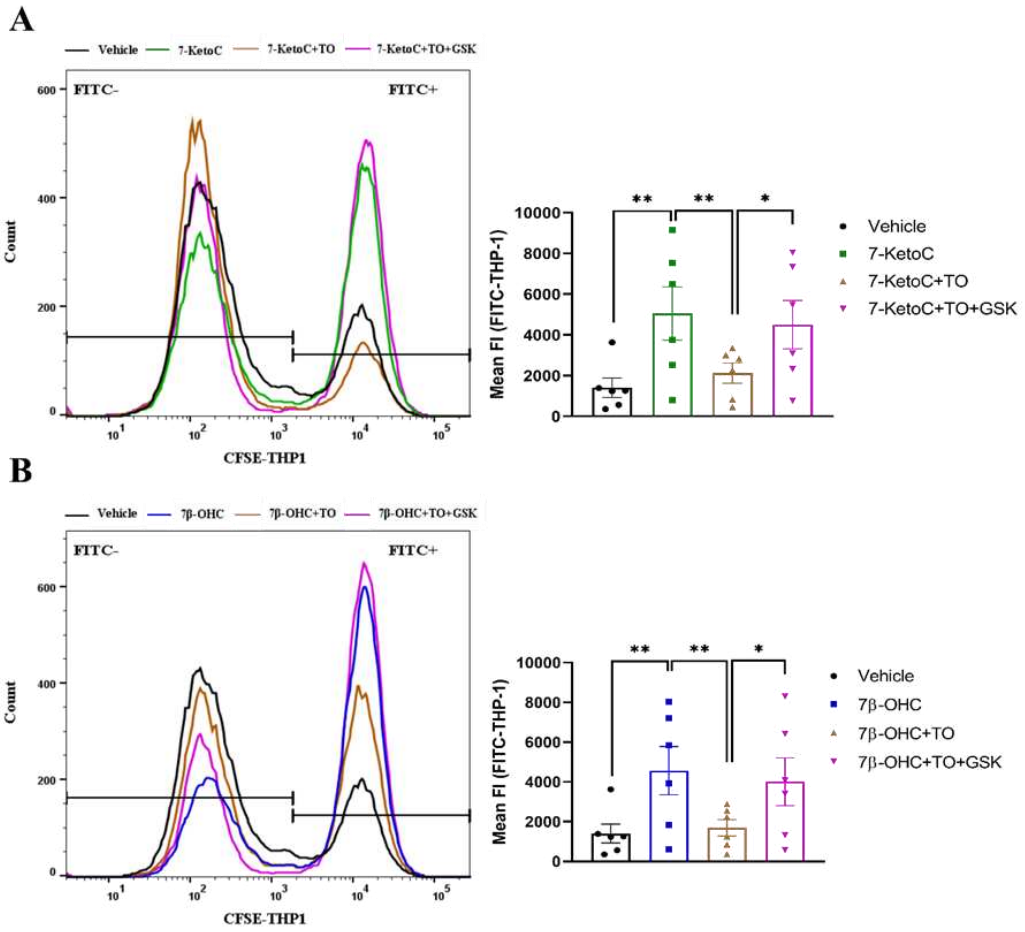

Figure 20: LXR activation lessened monocyte adhesion to fpEC in the presence of 7-ketoC or 7β-OHC

Pre-incubation with TO attenuated **(A)** 7-ketoC or **(B)** 7β-OHC induced attachment of THP-1 monocytes to fpEC (n=6). LXR inhibition by GSK antagonized the protective effects of TO. Data are presented as mean±SEM. Statistically significant differences between different treatments were calculated using one-way ANOVA followed by Tukey's post hoc test. *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

5. Discussion

During pregnancy, the mother's body undergoes significant physical and metabolic changes to support the growth and nourishment of the fetus. However, there is a risk of maternal maladaptation, where the body fails to adjust to these changes. Failure to adapt appropriately can lead to various pregnancy complications and have both short-term and long-term effects on the health of the mother and child (205). The placenta plays a pivotal role in mediating maternal adaptations during pregnancy by serving as a functional interphase between mother and fetus, separating their circulation (7). The placenta also produces hormones and growth factors that mediate maternal adaptations (206). Both gestational diabetes mellitus (GDM) and preeclampsia (PE) exhibit a shared characteristic of inflammation in the mother's systemic circulation. Additionally, placenta is exposed to inflammation during such conditions (12,207). Prolonged exposure to an inflammatory environment during pregnancy is a known risk factor for the development of cardiovascular and metabolic disease in both mothers and their offspring (12).

Endothelial cells are key players and master regulators of inflammation in systemic circulation, providing a constant anti-inflammatory and anti-coagulant state (208,209). Therefore, understanding the inflammatory processes that occur at the maternal-fetal interface in the placenta during gestation is crucial in determining the potential risks to both the mother and the developing fetus. This knowledge may help to develop strategies to prevent or minimize inflammation and reduce the risk of adverse health outcomes. Despite proper uteroplacental perfusion, fetoplacental vasculature dysfunction can lead to fetal abnormalities (210). Hence, gaining a deeper understanding of the function and maintenance of fetoplacental homeostasis could significantly impact fetal outcomes. Fetoplacental endothelial cells (fpEC) have received considerable attention in research due to their crucial role in maintaining cholesterol homeostasis, particularly in the context of GDM. Compared to healthy pregnancies, fpEC from GDM pregnancies show higher expression levels of LXR target genes, including ABCA1 and ABCG-1. Those upregulations of genes involved in cholesterol homeostasis allow a more efficient efflux of cholesterol to HDL (108). As the fetal blood comes into direct contact with fpEC, these cells play a crucial role in facilitating the delivery of cholesterol into the fetal circulation (149,211).

The initial objective of this study was to identify the inflammatory pathways elevated and endothelial dysfunction evoked in fetoplacental endothelial cells (fpEC) when exposed to 7-ketoC or 7β-OHC, whose levels are elevated in conditions of GDM (108). We found that they induce the activation of TLR4 pro-inflammatory pathway and are attributed to the inflammatory phenotype often observed in inflammatory pregnancy disorders. This study set out with the aim of assessing the significance of LXR activation against oxysterol-induced inflammation and endothelial dysfunction in fpEC. We discovered that in fpEC, pharmacological activation of LXR by the synthetic agonist T0901317 (TO) displayed attenuation of inflammatory responses and disruption of cell-barrier integrity triggered by oxysterols. Furthermore, our study reinforces the anti-inflammatory role of ABCA1, which is associated with its established capacity to efflux cholesterol.

Oxysterol levels are likely to be elevated in pregnancy disorders. They are implicated in oxidative stress and inflammation, which play an important role in placental pathophysiology (94). Previous studies from our lab measured the oxysterol levels in normal and GDM cord blood samples: 7α-OHC (22 ± 7 vs. 33 ± 14 ng/ml), 7β-OHC (15 ± 4 vs. 25 ± 17 ng/ml), 7-ketoC (36 ± 12 vs. 60 ± 27 ng/ml) (108). Several clinical studies reported levels of plasma oxysterols in the range of 30-40 ng/ml (79,80). However, oxysterol concentration in atherosclerotic plaques was increased by about 50 times higher (109,212). In preeclamptic placentas, the concentration of oxysterols present in atherotic lesions commonly observed in the chorioamnion and spiral arteries is likely much higher than normal plasma levels (112). Furthermore, they are known to have cytotoxic effects at higher concentrations in a variety of cell types (86,88). Therefore, we tested the cytotoxic effects of oxysterols on control and GDM fpEC at different concentrations in the first set of experiments. We observed cytotoxic effects in fpEC at a concentration of 20 μM and above. Based on those results, we decided to use a non-toxic concentration of 10 μM for all in vitro experiments, in order to mimic a pathophysiological condition that may likely occur. Additionally, our results nicely fit to the already published cytotoxic effects of oxysterols at higher concentrations in different cell types (86). Of note, we observed that the rate of apoptosis in GDM fpEC was significantly higher than that of control fpEC under unstimulated conditions. These findings indicate potential dysregulation in cellular survival mechanisms in the context of GDM (213).

Both 7-ketoC and 7β-OHC at 10 μM concentration upregulated mRNA expression of IL-6, IL-8, and ICAM-1 in both GDM and control fpEC. Additionally, in GDM fpEC, there was a significant induction of IL-1α and TNF-α mRNA. We discovered that the induction of the cytokine mRNA is the result of activation of MAPK and NFκB pathways downstream of TLR4. Furthermore, these two oxysterols induced the expression of ICAM-1 mRNA and protein in control fpEC. However, in GDM fpEC, both ICAM-1 and VCAM-1 were induced by the oxysterols. Interestingly, we found that only ICAM-1 but not VCAM-1 cell surface expression was significantly enhanced by oxysterols in both control and GDM fpEC. Although GDM fpEC have higher levels of oxysterols, an additional exogenous treatment did not have a significant impact on the overall inflammatory profile of the cells. The significantly higher BMI of GDM compared to the healthy one might have an influence on the elevated levels of VCAM-1 or some of the cytokine mRNA differently to control fpEC upon the exposure of oxysterols. Overall, the effects of oxysterols on GDM and control fpEC were comparable. We speculate that this could be due to the feedback mechanisms already exerted by the oxysterols via LXR activation in GDM fpEC which could potentially resist the further rise in inflammation upon exogenous triggers. Based on these results, we decided to study the underlying mechanism of inflammation or anti-inflammation solely in control fpEC.

Surprisingly, none of the cytokine mRNA induced by oxysterols in fpEC translated into corresponding increases in secreted cytokine levels, suggesting posttranslational regulation of protein synthesis. However, we observed a significantly increased concentration of cytokines in the supernatant of fpEC treated with LPS. It is widely recognized that LPS is a potent inducer of inflammation and capable of activating multiple TLR4 downstream signaling pathways, leading to the secretion of cytokines (214). Interestingly, we detected significant low levels of ICAM-1 cell surface expression in GDM fpEC compared to the normal. This result is consistent with the previously observed lower levels of ICAM-1 protein in GDM fpEC compared to the control group, which is likely due to post-transcriptional downregulation by specific miRNAs targeting ICAM-1 (215). The concept that alterations in the inflammatory marker profile in the mother do not always correspond to similar changes in the fetal circulation is well accepted (12). Levels of inflammatory mediators like leptin and cytokines such as IL-6, and TNF-α, were elevated in the serum of GDM mothers, while in neonates, their levels were not elevated (216). In such conditions, the placenta may act as a sensor for systemic inflammation by responding to a maternally derived environment and adapting to the changes to maintain normal placental

function (12). We hypothesize that oxysterols initiate a negative feedback loop that inhibits early inflammatory responses, such as cytokine release, through one of their receptors, such as LXR. Furthermore, we suggest that the ability of oxysterols to activate LXR is linked to the feedback mechanism, which may help to reduce cellular damage caused by oxysterols.

The fetoplacental vasculature regulates blood flow to the fetus by maintaining vasomotor tone (14). Throughout gestation, the fetoplacental vasculature acts as a barrier regulating transport of substances and pathogens to the fetus from maternal circulation (37). Therefore, the essential role of the fetoplacental endothelium is of paramount importance in the context of fetal growth and development, as it fundamentally governs the efficacious exchange of oxygen and vital nutrients at the interface where the fetal and maternal circulatory systems intersect (217). Pregnancy disorders such as GDM and PE are often associated with placental endothelial dysfunction and increased vascular permeability (218). These complications are also characterized by increased ROS generation, as well as mitochondrial dysfunction (66). In our study, we aimed to identify the role played by bioactive lipids, oxysterols, in contributing to the dysfunction of fetoplacental endothelial cells.

In the present study, we discovered that 7-ketoC or 7β-OHC at 10 μM concentration enhances the production of ROS in fpEC, one of the major signs of endothelial dysfunction (73). The induction of ROS by oxysterols can subsequently lead to the generation of ROS-derived oxysterols, which can further increase ROS production, creating a vicious cycle of oxidative stress and damage. Oxysterol-triggered generation of ROS is observed in a variety of cell types including cancer cells (219). In addition, previous studies from our lab documented the generation of reactive oxygen species (ROS) and the subsequent production of 7-ketoC and 7β-OHC in fpEC obtained from GDM cases in comparison to those from healthy donors (108). ROS disturbs mitochondrial bioenergetics and induces mitochondrial damage, including mutations in mitochondrial DNA, a leading cause of several pathologies, including neurodegenerative and cardiovascular diseases (176). ROS induce the decoupling of endothelial nitric oxide synthase (eNOS), leading to a subsequent decrease in the availability of nitric oxide (NO), and this deficiency is particularly pronounced in placentas affected by PE (220). In addition, mitochondrial dysfunction can cause increased ROS production, creating a vicious cycle of damage and dysfunction (221). In fpEC, 24 hours of 7-ketoC or 7β-OHC treatment triggered mitochondrial depolarization indicated by a drop in TMRM dye fluorescence. Despite this, fpEC did not undergo significant apoptosis at a concentration of 10 μM after 24 hours of

treatment. Nevertheless, the compromised mitochondrial function suggests that there is an impact on the cellular metabolic activity.

The rise in ROS levels of fpEC might be accompanied by changes in intracellular Ca^{2+}, as seen in other cell types. It has been shown that ROS activate L-type Ca^{2+} channels, resulting in a continuous flow of Ca^{2+} into the cells (222). ROS can activate Ca^{2+} channels in the plasma membrane or intracellular organelles, such as the endoplasmic reticulum and mitochondria, thereby elevating the cytosolic calcium concentration. On the other hand, calcium can increase the generation of ROS creating a vicious cycle leading to cell death if not regulated (223). The intricate interaction between Ca^{2+} and ROS is multifaceted and can have diverse consequences on cellular activities, notably affecting endothelial function (223). In our study, we observed a significant rise in calcium influx shortly after the start of the perfusion of oxysterols to the cells. While both oxysterols increased the concentration of intracellular calcium in fpEC, we observed a higher calcium flux with 7-ketoC exposure compared to 7β-OHC. Due to the rapid onset of calcium flux in response to stimuli, we limited our measurement of changes in calcium concentration to the first 10 minutes of addition with oxysterols. This is because downstream events typically occur after the initial calcium flux (224). It has been shown that both 7-ketoC and 7β-OHC possess the capability to promote Ca^{2+} influx by activating voltage-gated calcium channels (VGCC) or store-operated calcium channels (SOCE). However, the underlying molecular mechanism still needs to be understood (225). Also, further investigation is required to assess both the expression and functionality of these calcium channels in fpEC. Earlier research conducted on THP-1 monocytes has demonstrated that 7-ketoC can stimulate an increase in intracellular calcium concentration for up to 12 hours (226). The intracellular oxysterol-binding protein-related proteins (ORPs) ORP5 and ORP8 regulate Ca^{2+} signaling at membrane contact sites of the endoplasmic reticulum and the mitochondria (227). Most of these responses happen rapidly to necessitate changes in oxysterol-induced gene expression, thereby ruling out the involvement of LXR or other oxysterol receptors.

Elevated levels of cytosolic Ca^{2+} can drive actin stress fiber formation, resulting in subsequent dissociation of adherens junction and an increase in vascular permeability (177). Clearly, our results demonstrate that 7-ketoC and 7β-OHC affect cell junction integrity negatively by inducing the dissociation of VE-cadherin from the cell junction in association with rapid actin rearrangements. Additionally, real-time ECIS measurements indicated significantly reduced trans-endothelial barrier resistance. The rate at which calcium levels

change matches the rate at which the protective barrier holds up. 7-ketoC and 7β-OHC) cause a gradual and prolonged increase in calcium levels, resulting a delayed breakdown of the barrier. In contrast, thrombin triggers a quick and intense spike in calcium levels, causing a sudden drop in barrier integrity, which later recovers, similar to calcium signal. A previous study showed that fpEC isolated from GDM patients demonstrated reduced transendothelial resistance and disrupted actin organization (228). Based on our findings, oxysterols which are present at higher levels in GDM fpEC may be responsible for such a phenotype. Further, fpEC treated with 7-ketoC or 7β-OHC showed a significantly higher number of adhered monocytes compared to the control group, clearly a sign of endothelial activation and subsequent dysfunction. By taking together our results, it is plausible to predict that elevated levels of oxysterols may contribute to similar alterations in endothelial barrier function that are commonly observed in pregnancy pathologies.

In addition, we measured the elastic Young's modulus of fpEC by using atomic force microscopy. Our results showed that these two oxysterols increased the stiffness of the plasma membrane, which was reflected in Young's modulus value. It is one of the most commonly used parameters to describe the mechanical properties of plasma membranes. Increased Young's modulus indicates that the cell is stiffer and less deformable, which indicates decreased plasticity, which in turn affects the mechanical properties of the cells (229). This effect is thought to be due to the ability of oxysterols to incorporate into the membranes and to form aggregates with cholesterol and other lipids, leading to the formation of rigid domains within the membrane (98,99). Furthermore, a study by Shentu TP et al. showed that oxLDL and specifically 7-ketoC induce a decrease in the lipid order of membrane domains and are found to have an inverse correlation with endothelial stiffness and contractility (230). Additionally, some oxysterols can interact with membrane-associated cytoskeletal proteins and influence their organization and activity, affecting plasma membrane stiffness (231). Moreover, decreased deformability is commonly associated with increased cellular stress and altered cellular functions (232). The stiffness of endothelial cells affects their ability to transmit signals to smooth muscle cells, which control the contraction and relaxation of vessel walls, and thus impair the ability of the blood vessels to regulate blood flow. This can result in reduced blood flow and a variety of health problems, such as hypertension and cardiovascular disease (233,234).

In addition to the pro-inflammatory mediators that have been studied, the MAPK pathway has the potential to initiate the induction of various other inflammatory factors, such as COX-2, prostaglandins, matrix metalloproteinases, proteases, monocyte chemoattractant proteins, interferon-gamma, etc. (94,235,236). We did not access the expression of all those inflammatory mediators here as it was beyond the scope of this study. However, considering oxysterol to be a potent inducer of inflammation in fpEC, we explored strategies to mitigate this observed corroborated dysfunction in fpEC. Based on this background, we hypothesized that LXR could be a promising candidate for targeting cellular function in fpEC, as studies have shown the key role of LXR in the suppression of inflammation besides its well-studied role in the regulation of lipid and glucose metabolism (73). LXR can be activated endogenously by certain oxysterols and through pharmacological means using synthetic ligands. It has been shown that LXR activation inhibits the production of pro-inflammatory cytokines such as TNF-α and IL-6, while promoting the expression of anti-inflammatory cytokines such as IL-10 in macrophages and neuronal cells (154,237).

Consistent with the well-established anti-inflammatory effects of LXR activation, our findings indicated a significant reduction in oxysterol-stimulated phosphorylation of NFκB and MAPK (p-38, ERK, JNK) proteins in LXR-activated cells by T0901317 (TO). Furthermore, we demonstrate decreased cytokine transcription and ICAM-1 total and surface protein levels in cells pre-incubated with TO prior to oxysterol addition. In fpEC, we found that TO significantly increased ABCA1 protein expression, which is a target gene of LXR involved in cholesterol efflux, by 20 to 30-fold. ABCG1, which is also an LXR target cholesterol efflux gene was induced by TO to merely 1.7-fold in our cells. Previous studies in macrophages have shown that ABCA1, not ABCG1, is the primary cholesterol efflux transporter involved in suppressing inflammatory events (140). Given that treatment with TO significantly affects ABCA1 expression in fpEC, we hypothesized that ABCA1, rather than ABCG1, is involved in the LXR-mediated inhibition of inflammatory responses in our cells.

Changes in the lipid order of the plasma membrane can impair intracellular transmission of cell signaling, especially inflammatory signaling, which mainly involves the activation of membrane receptors located in the lipid raft region (238). ABCA1 induction enhances cholesterol efflux from the cells to apoA1 (152). Consequently, ABCA1 can efflux cholesterol from the plasma membrane and redistribute the cholesterol content of the membrane, altering its fluidity, consequently disrupting TLR4 activation at the plasma membrane locus (140).

However, the ability of ABCA1 to efflux oxysterols out of the cells cannot be entirely excluded here (239). Removal of 25-hydroxycholesterol from murine fibroblast cells efficiently by ABCA1 has been described (240). Whether 7-ketoC and 7β-OHC are trafficked out of the cells by ABCA1 remains a question and needs further in-depth studies. However, in macrophages, ABCG-1 promoted the efflux of 7-ketoC (241). To elucidate the involvement of ABCA1 in suppressing inflammation in fpEC, we introduced probucol, a cholesterol efflux activity inhibitor of ABCA1 (242,243). In fpEC pre-treated with probucol along with TO followed by oxysterol addition, the protective effects of TO were counteracted, and we observed a significant increase in inflammatory responses as created by 7-ketoC or 7β-OHC stimulation. These findings highlight the importance of the ABCA1-LXR axis in modulating inflammatory responses and suggest that targeting ABCA1 could be a potential target for regulating inflammation-related conditions in cells. While we propose that ABCA1 plays a critical role in LXR-mediated attenuation of inflammatory signaling in fpEC, we do not ignore the possibility that LXR may also repress inflammatory gene expression through trans-repression, a process that involves sumoylated LXR. Taken together, our findings highlight the significant potential of LXR as a target for inflammatory disorders in pregnancy. The protective effects of TO we observed in fpEC are similar to those demonstrated in placental trophoblasts previously by Aye I et al. (94).

It should be noted that while TO is a potent activator of LXR, it is also capable of activating the farnesoid X receptor (FXR), which is an essential metabolic regulator and plays a role in the regulation of inflammation (244). However, the effective concentration (EC_{50}) of TO for LXR is 50 nm, while EC_{50} for FXR is 5 μM. In our study, we propose that, at 2 μM concentration of TO, the effective activation of LXR is predominant. Our studies unravel the importance of ABCA1 in the regulation of inflammation in fpEC. Therefore, we hypothesize that the activation of LXR is the primary mechanism at play.

Some studies illustrate that oxysterols are able to activate toll-like signaling (94). Therefore, we investigated whether oxysterols induce inflammation by a TLR4-dependent mechanism. We used a drug developed for blocking TLR4 specifically called Tak-242 (245). Pre-treating cells with Tak-242 for 2 hours, followed by treatment with oxysterols significantly attenuated the inflammatory responses elicited by exposure to oxysterols. Tak-242 completely abolished the inflammatory responses induced by ultra-pure LPS which binds only to TLR4, confirming the efficiency of Tak-242 to inhibit TLR4 activation (246). Our findings indicate

that the effects of oxysterols on inflammation in fpEC are likely mediated through the activation of TLR4 signaling. Previous studies in placental trophoblasts have also demonstrated the ability of oxysterols to activate TLR4 (94).

In fpEC, the involvement of other TLRs in oxysterol-mediated induction of inflammation cannot be ruled out. However, our results demonstrate the major role of TLR4 in oxysterol-induced inflammation in placental endothelial cells. On the other hand, it is well known that certain oxysterols can activate LXR in a variety of cell types, including fpEC (73). 7-ketoC or 7β-OHC induced weak expression of ABCA1 in fpEC (108). As previously suggested by Aye et al., the pro-inflammatory responses observed in fpEC followed by oxysterol treatment may be the result of an imbalance between pro-inflammatory and anti-inflammatory signals (94). Here, we also postulate that the ability of oxysterols to activate LXR could be an outcome of negative feedback mechanisms that cells adopt to protect themselves from oxysterol-induced toxicity or inflammation via LXR-dependent mechanisms.

It is currently not fully understood how oxysterols activate TLR4, but it is believed that the activation process may involve the binding of oxysterols to the accessory molecule MD-2. Cholesterol is shown to bind to MD2 (247) and oxidized cholesterol ester has been shown to induce TLR4 dimerization by recruiting MD-2 (248). One can speculate that TLR4 activation by oxysterol may involve similar molecular mechanisms.

Besides suppressing inflammation, LXR activation has been demonstrated to play an important role in the maintenance of blood-brain barrier integrity in vitro. LXRα knockout in brain endothelial cells resulted in reduced tight junction protein expression and consequently, a more permeable barrier (141). Another study done by Elali et al. shows evidence that in the ischemic brain, LXR activation is associated with an enhancement of blood-brain barrier integrity and a decrease in the permeability by the activation of calpastatin that deactivates calpain-1/2, stabilizing p120 catenin to improve the tight junctions. Furthermore, LXR agonism has been shown to improve TNF-α-induced endothelial dysfunction in HUVEC (141,249). Our findings are consistent with the previously reported vascular protective effects of LXR.

While we did not observe significant alterations in mitochondrial membrane potential or calcium flux stabilization by LXR activation, we found that LXR activation significantly enhanced transmembrane resistance, as well as the organization of VE-cadherin and F-actin. Furthermore, activation of LXR in fpEC was found to decrease the occurrence of oxidative stress and monocyte adhesion induced by oxysterols. Reduced oxysterol-triggered monocyte

adhesion in LXR-activated cells is attributed to the reduction in ICAM-1 cell surface localization, as demonstrated earlier in the results. Previously, in human umbilical vein endothelial cells, reversal of lysophosphatidylcholine-induced monocyte adhesion upon LXR activation has been demonstrated (143). LXR participates in the regulation of ROS generation by inducing the expression of anti-oxidant genes such as nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor, which regulates the transcription of an array of anti-oxidant genes (199,200). While our study did not explore the exact mechanism of ROS homeostasis in LXR-activated fpEC, we hypothesize that the activation of LXR leads to an increase in the expression of antioxidant genes in these cells. LXR is known to play a role in the reversal of oxysterol-induced cytotoxicity and disruption of mitochondrial dynamics (198,250).

In fpEC, LXR activation failed to stabilize oxysterol-induced rise in intracellular Ca^{2+} concentration significantly. However, we still observed a trend in decrease of oxysterol-induced Ca^{2+} flux, but it failed to give significance statistically with 5 biological replicates. Interestingly, we observed a significant reduction in thrombin-induced ΔCa^{2+} (Maximum Ca^{2+}- Basal Ca^{2+} fluorescence) concentration in fpEC. The ΔCa^{2+} caused by thrombin was several folds higher than that induced by oxysterols and therefore, a 30% decrease in ΔCa^{2+} concentration in TO pre-incubated cells gave statistical significance. Our results show that LXR might also play a role in the stabilization of intracellular calcium concentration. Evidence relating to LXR and calcium homeostasis in endothelial cells is extremely limited. However, a study demonstrates that LXR agonists inhibit thrombin-dependent platelet activation, which involves G-protein coupled receptor (GPCR) activation and subsequent calcium mobilization. The study also found that the LXR ligand TO inhibited calcium mobilization stimulated by platelet collagen (251). The findings suggest that LXR activation could be a promising therapeutic approach to prevent platelet activation and reduce the risk of thrombosis. Nevertheless, it should be noted that this study focused specifically on platelets. Thus, additional research is necessary to examine the potential impact of LXR activation on intracellular calcium levels in other cell types and under different circumstances.

The role of LXR in maintaining blood-brain barrier integrity and function has been shown already (141). Whether LXR is involved in the maintenance of placental barrier integrity is not known. One of the major components of the placental barrier is the fetoplacental endothelium (211). Therefore, we wanted to investigate the implications of LXR activation on the barrier functionality of fpEC in the presence and absence of oxysterols. We observed

improved electrical resistance and VE-cadherin and actin organization in cells incubated with TO; an effect that was abolished by LXR antagonist GSK. Furthermore, oxysterol-induced drop in electrical resistance is significantly regained in LXR-activated cells. Of note, the proper VE-cadherin organization is regained in TO pre-incubated cells followed by oxysterol stimulation. Actin stress fiber formation also appeared to be less in those cells with active LXR. These findings emphasize the crucial role of LXR in preserving barrier function and suggest that LXR agonists could hold promise for enhancing barrier function in disease-related situations. However, it's worth noting that TO did not have a significant impact on the increased cellular stiffness induced by oxysterols. Contemporary research suggests that diminishing the cholesterol content in the plasma membrane leads to a decline in lipid arrangement within the membrane, consequently augmenting its stiffness (230). However, taken together our results indicated the active role of LXR in maintaining cell junction integrity. The underlying molecular mechanism by which LXR signals to trigger the cell junction remains to be investigated.

6. Conclusions

My thesis highlights the effects of 7-ketoC and 7β-OHC on placental inflammation and the underlying mechanisms involved. The stimulatory effect of oxysterols on MAPK and NFκB signaling, as well as on the mRNA expression levels of IL-6 and IL-8 and total cellular ICAM-1 protein expression was demonstrated in fpEC. The results of this study displayed a crucial role of TLR4 in instigating inflammation by oxysterols in fpEC. Consequently, function of the fetoplacental barrier was impaired in the presence of oxysterols. Mechanistically, it is shown that LXR activation attenuated oxysterol-induced pro-inflammatory signaling and endothelial dysfunction, thus enhancing intercellular barrier integrity. We also demonstrate that ABCA1 as a cholesterol efflux transporter contributes to LXR-mediated suppression of inflammation. The summary of the results is depicted in Figure 21. Although the mechanism of LXR has been extensively studied and the potential therapeutic application of LXR agonists in numerous disorders including atherosclerosis, diabetes, and cancer, have been investigated, the action of oxysterols and the role of LXR in the fetoplacental vasculature are novel.

Taken together, these results have important implications for the understanding of placental inflammation and endothelial dysfunction and their underlying mechanisms as

elevated oxysterol levels may contribute to certain disorders in pregnancy. While oxysterols possess the ability to activate LXR, they can also induce inflammation and vascular dysfunction at pathophysiological concentrations, thereby contributing to the development and progression of placental pathologies. Furthermore, as fpEC play a crucial role in controlling the transfer of substances and inflammatory signals from the mother to the fetus, our findings are of particularly relevant to conditions related to placental inflammatory disorders. Therefore, our study adds to the body of scientific knowledge in this area and has the potential to inform future research and clinical practice. These findings may have clinical relevance as oxysterols may serve as a diagnostic biomarker for pregnancy disorders such as GDM and PE. In addition, our study highlights the essential role of LXR in maintaining placental vascular homeostasis by regulating inflammatory events and preserving endothelial barrier integrity. This suggests that LXR may be a promising therapeutic target for such disorders.

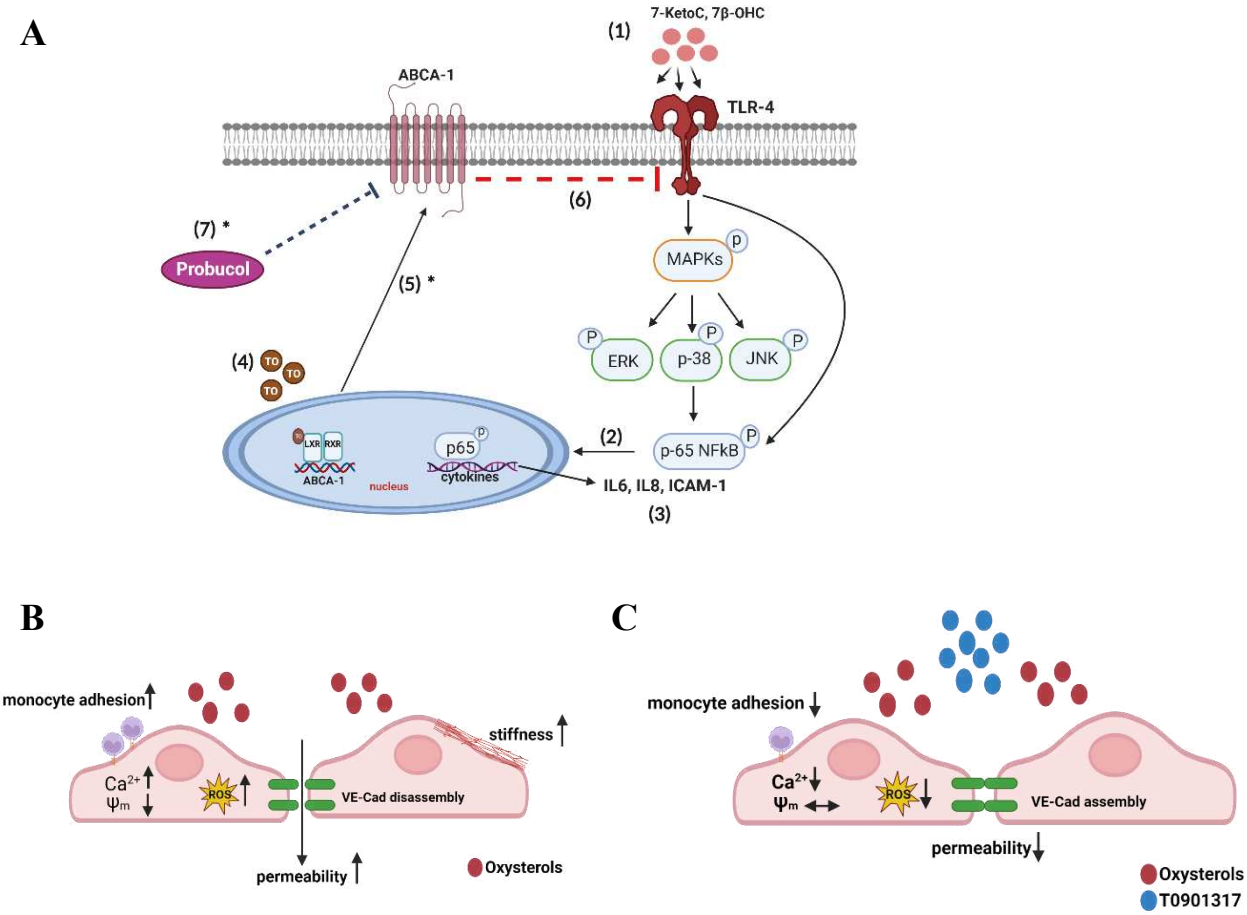

Figure 21: **(A)** The proposed mechanism of ABCA1-mediated inhibition of TLR4 activation by oxysterol in human fetoplacental endothelial cells. (1) When fpEC are exposed to 7-ketoC and 7β-OHC, the TLR4 signaling cascade is activated, leading to concurrent phosphorylation of MAPK (JNK, p38, ERK) and p-65 NFκB. (2) Following phosphorylation, the p-65 NFκB complex translocates to the nucleus. (3) In the nucleus, the p-65 NFκB complex initiates the transcription of inflammatory mediators such as IL-6, IL-8, and ICAM-1. (4) Treatment with T0901317 activates LXR/RXR heterodimer, which subsequently induces ABCA1 expression. (5) ABCA1 then translocates to the plasma membrane, triggering membrane cholesterol efflux and altering membrane cholesterol homeostasis (140). (6) Depletion of cholesterol from the plasma membrane disrupts TLR4 activation by oxysterols (94). Probucol inhibits ABCA1-mediated cholesterol efflux in fpEC (149) and hence favoring oxysterol to activate TLR4 signaling while membrane cholesterol is maintained. *already shown in fpEC by others (108,149) . The figure is reproduced from (1) with the permission of publisher. **(B)** 7-ketoC and 7β-OHC alter the functionality of fpEC by triggering ROS generation, calcium mobilization, mitochondrial depolarization, monocyte adhesion and disassembly of VE cadherin from the intercellular junction causing increased vascular permeability. **(C)** Pre-activation of LXR using T0901317 in fpEC attenuated oxysterol-induced ROS generation, calcium mobilization, monocyte attachment and VE-cadherin disassembly, while having no effect on the mitochondrial depolarization induced by oxysterols.

7. Study limitations

In our study, we acknowledge certain limitations that provide valuable insights for future research. A notable limitation is the small sample size of our pilot cohorts, which requires further validation in larger cohorts to increase the significance of the results. Moreover, results and extrapolated conclusions are meaningful for the end of pregnancy, as we used the term fetoplacental endothelial cells. This limitation highlights the need to expand our investigation to include fetoplacental endothelial cells from different stages of pregnancy to gain a comprehensive understanding. Additionally, we cultured fpEC at 21% O2 and we recognize that culturing the arterial fpEC at hypoxic conditions, approximately 12% O2, would be more physiological as it better mimics the cell environment in utero, where fpECs are in contact with deoxygenated blood. Consequently, this experimental adjustment would provide more accurate and meaningful results. This study is limited in its ability to provide a deeper understanding of the post-transcriptional regulation of cytokines in oxysterol-treated cells, which could explain the discrepancy between mRNA and protein results. A proteomic approach should be used to further elucidate the effects of oxysterols. This will allow the identification of inflammatory factors induced at the protein level, providing a comprehensive understanding of the cellular responses upon exposure to oxysterols and filling the gaps in our current understanding and potential therapeutic implications. Overall, addressing these limitations and capitalizing on the strengths of our study will pave the way for more comprehensive and robust research in this area.

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9. Appendix

Preparation of Buffers

Western blot sample loading buffer

1x loading buffer from Biorad Cat#1610791
1x reducing agent from Biorad Cat# 1610792
Ripa Buffer

Western blot Running Buffer (10x)

30.3 g Tris
144.0 g Glycine
10.0 g SDS
Make upto 1 Litre with ddH2O and store at RT

TBS-T (1x, 1 L)

20 ml Tris, 1 M, pH 7.5
100 ml NaCl, 2.5 M
1 ml Tween 20
Make upto 1 Litre with ddH2O and store at RT

Blocking solution (5%; 20 ml)

1 g non-fat milk powder
20 ml TBST

2% Gelatin stock solution

10g Gelatin
500 ml 1x HBSS
Autoclave the solution
10 ml gentamicin
Store in fridge and dilute 1:2 in 1x HBSS before use

T0901317 stock solution (5 mM)
Add 2.07 ml absolute ethanol to 5mg powdered T0901317 to obtain 5 mM stock. Store at -20°C.

7-ketocholesterol stock solution (10 mM)
Add 1248 µl ethanol absolute to 5 mg of 7-ketoC to obtain 10 mM stock. Store at -20°C.

7β-hydroxycholesterol stock solution (10 mM)
Add 1248 µl ethanol absolute to 5 mg of 7β-OHC to obtain 10 mM stock. Store at -20°C.

Probucol (10 mM)

Weigh 10 mg of probucol and add 1934 µl of DMSO to obtain 10 mM stock. Store at -20°C.

GSK2033 Stock solution (10 µM)
Add 845 µl of DMSO to 5 mg of GSK2033 to obtain 10 mM stock. Store at -20°C.

Experimental storage buffer

2 mM Ca2+
138 mM NaCl
1 mM $MgCl_2$
5 mM KCl
10 mM HEPES
2.6 mM NaHCO
0.44 mM KH_2PO_4
amino acid, and vitamin mix
10 mM glucose
2 mM L-glutamine
1% Penicillin/Streptomycin
1% Fungizone
Adjust pH to 7.4 and store at RT

Physiological Buffer

2 mM Ca2+
135 mM NaCl
1 mM MgCl2
5 mM KCl
10 mM HEPES
10 mM glucose
Adjust pH to 7.4 and store at RT

Table 3: Chemicals used for cell culture experiments

Product	**Company**
Endothelial cell growth media	Promocell
Endothelial cell growth Supplements	Promocell
Gelatin	Sigma-Aldrich
Penicillin/ Streptomycin/ Gentamycin	PAA Laboratories
HBSS	Gibco
Trypsin-EDTA	Gibco
T0901317	Sigma-Aldrich

7-ketocholesterol	Cayman Chemicals
7β-hydroxycholesterol	Sigma-Aldrich
GSK3033	Cayman Chemicals
Probucol	Sigma-Aldrich
TMRM	Thermo Fisher Scientific
Fura-2 AM	Thermo Fisher Scientific
LDH cytotoxicity kit	Thermo Fisher Scientific
BCA	Thermo Fisher Scientific
XT Sample loading dye	Biorad
XT reducing agent	Biorad
Nitrocellulose membrane	Biorad
Polyacrylamide gel	Biorad
Gel transfer Stack	Biorad
Chemiluminescent reagent	Biorad
Not-fat milk powder	Biorad
Stripping Buffer	Thermo Fisher Scientific
Bovine Serum Albumine, 2 mg/ml	Thermo Fisher Scientific
Protease Inhibitor Cocktail tablets	Sigma-Aldrich
MOPS Buffer	Biorad
Phosphatase inhibitor	Sigma-Aldrich
Cytokine Multiplex Kit	Thermo Fisher Scientific
RNA isolation Kit	QIAGEN
Luna cDNA Kit	New England Biolabs

Luna qPCR reagent	New England Biolabs
Syber Green	Bio-Rad
High Capacity Reverse Transcriptase Kit	Life technologies
DNA ladder	New England laboratories
Nuclease free water	Invitrogen
RIPA Buffer	Sigma-Aldrich
TAk-242	Sigma-Aldrich

Table 4: Antibodies used for western blotting (WB), immunofluorescence (IF) and Flow cytometry (FC)

Antibody	**Working dilution**	**Catalogue Number and Company**
ABCA1 (WB)	1: 2000	ab18180, Abcam
ABCG1 (WB)	1:500	sc-20795, Santa Cruz
p-p42/44 MAPK (WB)	1: 2000	9101, Cell signaling Technology
p42/44 MAPK (WB)	1: 2000	9102, Cell signaling Technology
p-JNK (WB)	1: 2000	9251, Cell Signaling Technology
JNK (WB)	1: 2000	9252, Cell Signaling Technology
p-p38 MAPK(WB)	1: 2000	9211, Cell Signaling Technology
p38 MAPK (WB)	1: 2000	9212, Cell Signaling Technology
p-p65 NFkB (WB, IF)	1: 2000, 1:250	3033, Cell Signaling Technology
p65 NFkB (WB)	1: 2000	8242, Cell Signaling Technology
ICAM-1 (WB, IF)	1:2000, 1:250	ab109361, Abcam
VCAM-1(WB)	1:1000	ab98954, Abcam
β-actin	1:2000	2125, Cell Signaling Technology
α-tubulin	1:2000	2125, Cell Signaling Technology
VE-Cadherin (IF)	1:250	sc-9989, Santa Cruz
CD54 ICAM-1 (FC)	1:20	353107, Biolegend
CD106 VCAM-1	1:20	305809, Biolegend