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Alteration of Barrier Function in Gestational Diabetes Mellitus

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Abstract

Gestational Diabetes Mellitus (GDM) is associated with changes in nutrient and oxygen supply of the fetus. This leads to certain consequences for infants like fetal macrosomia, increased placental weight, and an increased risk for cardiovascular disease of the offspring that occur later in life. We hypothesized that the diabetic environment of GDM may affect feto-placental endothelial barrier function. To this end, the barrier function of primary arterial and venous feto-placental endothelial cells (AEC, VEC) isolated after uncomplicated and GDM pregnancies was compared by monolayer impedance measurements using the ECIS system. This research shows increased monolayer impedance in GDM derived AEC (n=9; +17.8%; $p<0.05$) compared with AEC from normal pregnancies (n=6). In contrast to that, GDM derived VEC formed monolayers with lower impedance (n=5; -23.4%; $p<0.05$) compared to normal VEC. Gene expression analysis of genes involved in barrier function RT-qPCR showed increased α catenin expression (+81%; p=0.02) in GDM AEC (n=4) when compared to normal AEC (n=3). Concomitantly, in GDM VEC (n=3) a decreased expression of claudin-7 (-55%; $p<0.02$) was detected when compared to normal VEC (n=3). Western blot analysis revealed claudin-5 protein increase (+33.6%; $p<0.02$) in GDM VEC (n=6). Compared to normal VEC (n=6) VE-cadherin was reduced (-51%; $p<0.05$) in GDM. In GDM AEC (n=5) tight junction protein 1 was increased (+181%; $p<0.01$). In order to investigate whether changes in barrier function and junctional molecule expression is a result of long term hyperglycaemia, normal VEC (n=4) and GDM derived VEC (n=3) were exposed to hyperglycaemia (15 mM D-glucose) for three days, and impedance was measured. This treatment caused lower impedance of GDM VECs when compared to the L-glucose treated control group. This effect maintained after cells were transferred to normal culture conditions again for another three days. By contrast, long term hyperglycaemic treatment of normal VEC did affect barrier function. We can conclude that hyperglycaemic environment alter feto-placental endothelial function. Hyperglycaemia is one component causing the changes, but other factors will contribute.

Zusammenfassung

Gestationsdiabetes (GDM) ist mit metabolischen und anatomischen Veränderungen der Plazenta assoziiert, was in einer ineffizienten Nähr- und Sauerstoffversorgung des Fötus resultiert. Dies führt zu Komplikationen, wie einem erhöhtem Plazentagewicht; Makrosomie des Kindes und einem erhöhten Risiko, für das Kind im Erwachsenenalter an Herz-Kreislauf Erkrankungen zu erkranken. Es ist anzunehmen, dass eine diabetische Umgebung Auswirkungen auf die Barrierefunktion der feto-plazentalen Endothelzellen hat. Zu diesem Zweck wurden arterielle und venöse Endothelzellen aus der Plazenta nach unauffälligen und nach diabetischen Schwangerschaften isoliert und die elektrische Impedanz mittels ECIS gemessen. Außerdem wurden Proteine, die Zell-Zell Interaktion vermitteln, auf mRNA und Proteinebene gemessen. Die Impedanz des Monolayers von arteriellen Endothelzellen (AECs) aus diabetischen Schwangerschaften (n=9) war im Vergleich zu normalen Schwangerschaften (n=6) erhöht (+17.8%; $p<0.05$). Im Kontrast dazu zeigen diabetische venöse Endothelzellen (VECs) eine niedrigere Impedanz (n=5; -23.4%; $p<0.05$) im Vergleich zu VECs aus unauffälligen Schwangerschaften (n=5). Die qPCR wies eine erhöhte Genexpression von α Catenin (+81%; $p=0.02$) in diabetischen AECs (n=4), im Vergleich zu nicht diabetischen AECs (n=3) auf. In diabetischen VECs (n=3) wurde hingegen eine erniedrigte Expression von Claudin-7 (-55%; $p<0.02$) im Vergleich zu normalen VECs (n=3), gemessen. VEC nach diabetischen Schwangerschaften (n=6) zeigten eine erhöhte Proteinexpression von Claudin-5 (+33.6%; $p<0.02$) und eine erniedrigte Expression von VE-cadherin (-51%; $p<0.05$) als VECs nach normalen Schwangerschaften (n=6). In diabetischen AECs war die Proteinexpression von Tight junction protein 1 (+181%; $p<0.01$) erhöht. Um festzustellen, ob die Veränderungen in der Barrierefunktion das Resultat einer länger andauernden Hyperglykäemie sind, wurden normale (n=4) und diabetische VECs (n=3) für drei Tage in einer hyperglykämischen Umgebung (15 mM D-Glukose) inkubiert und danach die Impedanz gemessen. Im Experiment zeigte sich eine erniedrigte Impedanz bei diabetischen VECs, im Vergleich zur Kontrollgruppe die mit L-Glukose inkubiert wurde. Dieser Effekt blieb stabil, auch nachdem die Zellen wieder für drei Tage unter normalen Kulturbedingungen kultiviert wurden. Daraus lässt sich schließen, dass eine erhöhte Glukosekonzentration in der Umgebung Auswirkungen auf die Barrierefunktion der feto-plazentalen Endothelzellen hat. Die Hyperglykämie dürfte aber nur einer der vielen Faktoren sein, welche die Barrierefunktion der Endothelzellen der Plazenta beeinflussen.

1. Introduction

1.1. Placental structure

The human placenta is a villous and highly vascularized organ with a disk-like, flat appearance. It consists of the chorionic plate, intervillous space and basal plate. The chorionic plate consists of fetal blood vessels that branch from the umbilical cord. The basal plate is connected with the maternal side, i.e. the uterus with the spiral arteries that fill the intervillous space with blood. This space includes the main functional units of the placenta, the villous tree. These structures are built up of four membranes, the outer syncytiotrophoblast, an underneath located layer of cytotorophoblasts, connective tissue of the villous, and the feto-placental endothelium. In these villi the exchange of nutrients and oxygen between maternal and fetal circulation takes place. Two umbilical arteries transport oxygen-deficient blood and waste products from the fetal metabolism to the feto-placental vasculature inside the villi which bath in maternal blood. There, the fetal blood is enriched with oxygen and nutrients and drains back through endometrial veins and umbilical vein to the fetus (Gude et al. 2004).

1.2. Development of the human placenta

The placenta is a highly vascularized organ. Therefore, development, branching and maturation of a complex vascular system are necessary to perform adequate exchange of nutrients and oxygen from maternal to fetal circulation. This vascular development occurs in two steps, vasculogenesis, followed by angiogenesis.

Vasculogenesis defines the process of formation of new blood vessels achieved by hemangioblastic stem cells in the first weeks of gestation. The following step, angiogenesis, involves formation of blood vessels from pre-existing ones and the maturation of a capillary network (Lopa Leach & Mayhew 2005).

1.2.1. Vasculogenesis

Early placental vessel development starts around day 21 post conception (pc). In this first stage primary villi are formed. This structure consists of an outer layer of syncytiotrophoblast, a connected monolayer of cytotrophoblast and a core, filled with mesenchymal stem cells. The primitive mesenchymal villus consists of a dense and cell-rich stroma with only small vessels (Huppertz 2013). Development into a secondary villus occurs as pluripotent stem cells aggregate, differentiate and migrate to form haemangioblastic cords (Lopa Leach & Mayhew 2005). Enlargement of centrally located intercellular clefts also happens at this stage. Fusion of these clefts results in formation of endothelial tubes with a larger lumen. Clearly defined,

long, polygonal lumina of heaemangioblastic cords with surrounding endothelial precursor cells (angioblasts) develop at around day 28 pc (Kaufmann et al. 2004). Further, haemangioblastic stem cells of the cord undergo morphogenesis to develop and change their organization. As a result of this, the inner layer of the cord is built of hematopoietic cells, whereas the outer layer is formed by angioblastic cells. These angioblastic cells will mature into endothelial cells later. To form a vascular network, branching of vessels and extension of a paracellular cleft between angioblastic cells is required (Lopa Leach & Mayhew 2005; Demir et al. 2007; Charnock-Jones et al. 2004). Anatomic connection and a fully functional circulation, between cord and embryo is established between day 32 and 35 pc (Kaufmann et al. 2004).

Vascular development is highly regulated by pro- and anti-angiogenic factors. In vasculogenesis, aggregation, stimulation of growth and development of haemangioblastic stem cells is regulated by vascular endothelial growth factor (VEGF). This key molecule of vascular development is highly expressed in early pregnancy by villous trophoblast and stromal macrophages. Furthermore, differentiation of haematopoietic and angioblastic cells is also regulated by VEGF and fibroblast growth factors (FGFs) (L Leach & Mayhew 2005).

1.2.2. Angiogenesis

At day 32 pc until delivery the terminal stage of placental development takes place. In this phase the placenta is formed by a mixture of villi at different stages of maturation. Consequently, relation and interaction between chorionic villi and trophoblastic layers influence each other's functions in a paracrine manner. During villi maturation, angiogenesis is divided into two different types, branching and non-branching angiogenesis. Latter occurs after extension of existing endothelial tubes by proliferation, or after incorporation of endothelial progenitor cells along the vessel length. Branching angiogenesis occurs by the formation of new side branches, and is also called sprouting angiogenesis, as it builds up a capillary network. Early vessels are composed of endothelial tubes with an incomplete layer of pericytes (Charnock-Jones et al. 2004).

A villus, as a component of the vascular tree, can be divided into three parts: the stem villous as a base out of which the intermediate villi branches and at the tip of the villous, the terminal villi. Formation of the stem villous starts around week 15 pc, when diameter of villi increases. The forerunners of venous and arterial vessels, but also precursor smooth muscle cells

(SMCs) are formed. As a next step, the villous advances in a radial manner to build a paravascular capillary network. Worth mentioning is that, cytotrophoblast number reduces around week 16 pc. Consequently, the villi divide and become very thin, whereby the distance between intervillous space and fetal vessels gets smaller. Starting from week 25 pc till term, the development of villi switches to a non-branching longitudinal growth, starting from the tips of pre-existing ones, to form intermediate villi. Also, a decrease in proliferation of the trophoblasts and increase of endothelial proliferation is stimulated, as a result of vascular elongation. Terminal villi are formed as a result of capillary coiling that depends on the growth rate of capillaries. This matured villus finally consists of an extremely thin cover of trophoblasts, supplied by one or two capillary coils. The exchange of gases and nutrients from the mother to the fetal circulation occurs on the surface of this structure (Kaufmann et al. 2004).

As early placental vessels starts branching, the expression of VEGF rises. Consequently leading to the hypothesis that binding of VEGF to its receptors stimulates branching angiogenesis. Stimulation and regulation of this process is additionally mediated by a variety of proangiogenic factors like FGF, erythropoietin, leptin, adiponectin, placental growth factor, angiopoietins and the insulin/insulin like growth factor. Neighbouring cells, i.e. trophoblasts, macrophages and smooth muscle cells produce these factors. Worth mentioning is that hypoxia, as a main regulator of angiogenesis, also affects the expression of many proangiogenic factors (Cvitic et al. 2014).

1.3. Arterial versus venous endothelial cells

Vasculogenesis and angiogenesis are important to regulate functions of endothelial cells (ECs) to adhere to each other and built new vascular structures (Leach et al. 2002). The vascular endothelium, developed from the mesoderm, covers the vessels from inside. Among other things this dynamic system controls vascular tone, blood fluidity and also trafficking of blood cells and nutrients. In adaptation to different functions the ECs differ widely in morphology.

In principle all blood vessels share the same basic structure: the outer layer *tunica externa*, a middle layer *tunica media* and the innermost *tunica intima*. The first, *tunica externa* consists of connective tissue and SMCs to lend structural support. This layer represents the main part of veins and venules, whereas in arteries it reaches only half of wall thickness of veins. In contrast to veins, where this layer practically does not exist, the main compartment of arteries

is *tunica media*. It contains of multiple layers of longitudinally oriented SMCs and elastic material. In contrast to *tunica intima* as innermost layer of vessels which is composed of supportive matrix of connective tissue, like collagen and elastic fibers. The main difference concerning this layer is the presence of venous valves, a structure that prevents backflow of blood in veins.

ECs are typically flat, with a height from 0.2 µm to 3 µm. In general arterial endothelial cells (AECs) are thicker than venous and have a long and narrow structure. Venous endothelial cells (VECs) are short and wide, because the blood flow rates in veins are much lower. Normally, veins are larger in diameter but have thinner walls than arteries.

Another characteristics of morphological distinction between ECs are differences in junctional structures of cell-cell contact in the paracellular cleft. In this case, junctions in arteries are much tighter than those in veins, while there are just loosely or poorly organized junctional structures visible. Latter affects priory the tight junctional structures.

Diversification of the vessels can be determined at molecular level in expression of specific signalling molecules, ligands, receptors and transcription factors. For instance, VEGF is known to induce migration and survival of ECs, is required for development of embryonic vasculature, regulates arterial differentiation and increases permeability in tumour vessels (dela Paz & D'Amore 2009). It affects endothelial transport properties, depending on vessel types and underlying cell signalling pathways (Chang et al. 2000). Further it is known that the notch signalling pathway is necessary to rescue arterial and suppress venous differentiation (dela Paz & D'Amore 2009). Additional, it has been reported that nitric oxide also inhibits cell proliferation of ECs in a time and dose dependent manner (Gooch et al. 1997).

1.4. Junctional structures

Its important to be clear that junctional maturation as a very complex process, requires coordinated expression, localization and interaction of transmembrane proteins with linker molecules and cytoskeletal adaptor molecules (Kluger et al. 2013). Direct interactions between cells and extracellular matrix are important to permit a response to environmental changes. Therefore, junctional structures are essential to link cells to each other by transmembrane adhesive proteins. These proteins again interact with intracellular partners which are anchored to the cytoskeleton and stabilize the junctional structures (Bazzoni & Dejana 2004). One of the main features of these junctions is a selective permeability of molecules of a specific size, and this is termed endothelial barrier. At present only two major

pathways to transport specific molecules through this barrier are known: the transcellular and paracellular transport. The term transcellular transport refers to a vesicle-mediated moving of macromolecules through the cell. In contrast to paracellular pathways which is known as controlled transport of small molecules along the intercellular space between connected cells. It allows transport of molecules of less than 3 nm in diameter by so called endothelial junctions (Vandenbroucke et al. 2008).

Vascular permeability is regulated by inflammatory- and growth factors that induce or decrease vascular leaking. Thus, bradykinin, VEGF, histamine, tumor necrosis factor α, lipopolysaccharides, serotonin, thrombin and oxidants might increase permeability. As stabilizing factors, adenosine and the phospholipid sphingosine 1-phosphate were identified (Vestweber et al. 2008; Feng et al. 1996; Chang et al. 2000). Furthermore, a decrease of permeability, induced by junctional reorganisation, occurs as a result of shear stress (Seebach et al. 2007; Cummins 2012).

On the one hand it is now clear that the permeability of one cell can be influenced by multiple factors. But on the other hand exist dynamic, connecting structures between endothelial cells to facilitate responses to environmental changes. These structures are termed adherens junctions (AJ) and tight junctions (TJ). The junctions control special features of ECs, including vascular homeostasis (permeability to plasma solutes, leukocyte extravasation and infiltration into inflamed areas, regulated opening and closing of cell-to-cell contacts) and transfer of intracellular signals that influence growth and apoptosis of cells. Compared to epithelial cells, ECs shows no specific arrangement between TJ and AJ in the paracellular cleft. As a result of specific function of the endothelium, the organization of junctions varies along the vascular tree. For instance, the brain requires strict control of permeability and well-developed junctional structures, consequently it is rich in TJ. In contrast to post capillary venules allow dynamic trafficking of plasma and cells, and reveal poorly organized TJ (Bazzoni & Dejana 2004).

1.4.1. Adherens Junctions

AJs are major structures at the edge of cells in the endothelial barrier. These structures are in dynamic equilibrium and recycle continuously between the plasma membrane and intracellular compartments. AJs are essential to enable cells to adhere and communicate with each other and respond after environmental changes. Cell-cell adhesion is a selective process; therefore, only specific cells of the same tissue are able to connect to each other. This can be mediated by complexes of cell adhesion molecules (CAMs) that belong to the group of

transmembrane receptors. CAMs can be divided into three domains with specific properties: an intracellular domain to interact with the cytoskeleton, a transmembrane domain and an extracellular domain that links with other CAMs in a hemophilic or heterophilic manner, or to the extracellular matrix. Adhesion molecules are classified into four groups with different features: selectins, integrins, Ig superfamily members and cadherins. The main components that form AJ belong to the cadherin family, which are expressed in lymphatic and blood vessels. Cadherins mediate stable homophilic interactions between extracellular matrix and cytoskeleton, stabilizing neighbouring cell membranes by anchoring them to another (Alberts et al. 2008).

Vascular endothelial (VE)-cadherin (~115kDa) is specific to endothelial cells of blood vessels. It is expressed during development and is randomly distributed along the vascular tree (Dejana et al. 2008). Furthermore, VE-cadherin is important to initialize the development of a primitive vascular network into a mature vessel system. It is essential to stabilize EC contacts, and most mechanisms that affect vascular permeability, also interact with VE-cadherin (Vestweber et al. 2008). Binding to other proteins occurs under the influence of calcium that acts as a co-factor. With its C terminus VE-cadherin is linked to p120, β-catenin and γ-catenin (plakoglobin) that affects its activity. These catenins further bind α-catenin, which interacts with several actin-binding proteins, like tight junction protein 1 (TJP1 or ZO-1), α-actinin and others. By this way, α-catenin is bound to the cell membrane to mediate cell-cell contact, but when α-catenin associates to the actin cytoskeleton it leads to actin bundling. A sudden increase in stabilization of actin-AJ-complex occurs when α-catenin binds another α-actin and/or vinculin (Dejana et al. 2008). To accomplish that, a sufficient level of α-catenin is required for actin branching, as it supports anchoring of cadherin to the cytoskeleton. It seems likely that VE-cadherin is indirectly involved in formation of TJs as it stimulates expression of claudin-5. Increased cell-cell adhesion was further mediated when VE-cadherin binds to the receptor-protein tyrosine phosphatase VE-PTP that reduces VEGF-induced phosphorylation of VE-cadherin. Furthermore turns out that factors like histamine, thrombin, tumor necrosis factor-alpha and VEGF were detected to increase tyrosine phosphorylation of cadherin-catenin components and thus, to increase permeability of ECs (Vestweber et al. 2008).

TJP1 usually belongs to the group of TJ-proteins, but contrary it is also found in AJs during the early phase of cell-cell connection. In this stage it is linked via α-catenin onto the cell

membrane, whereas during placental development it subsequently switches to TJs. However, association of VE-cadherin with catenins is required for cellular control of endothelial permeability and junction stabilization along the vascular tree. Furthermore, organization of junctional structures also varies dramatically in different stages of cell confluency. Thus the control of permeability is better in long-confluent cultures than in sparse cells. Worth mentioning is that signalling molecules (VEGF, GTPases, IQGAP1, PECAM), permeability-increasing agents (thrombin, histamine, plasmin) and enzymes (MMP, PI-3-kinase) can influence the architecture of AJs (Bazzoni & Dejana 2004).

1.4.2. Tight Junctions

TJs are formed during vasculogenesis (Kaufmann et al. 2004) and are located in the paracellular cleft forming a very close cell-cell contact in epithelial and endothelial cells. Therefore, TJ create a 4 nm gap (Lopa Leach & Mayhew 2005) to allow regulated diffusion of water, solutes, larger molecules and other cells, across intercellular spaces. TJs provide structural integrity to tissues and create polarized barriers to regulate movement of membrane molecules between apical and basolateral plasma membrane. It is known that TJs are very dynamic structures that regulate cellular differentiation, signal transduction, proliferation, migration and gene expression. In arteries these structures are well developed, compared to the loosely organization in venous vessels (Bazzoni & Dejana 2004; Cummins 2012). TJs consist of a complex arrangement of cointeracting transmembrane and intracellular proteins. While occludin, claudin and junctional immunoglobulins (JAM) belong to the transmembrane domain proteins, tight junction proteins (TJP1, TJP2 and TJP3) and cingulin are parts of intracellular molecules that are linked to the cytoskeleton (Bazzoni & Dejana 2004).

Occludin is a 65 kDa transmembrane protein localized in endothelial and epithelial cells. It mediates intercellular adhesion of confluent cells, might induce cell aggregation and plays a role in TJ stability and barrier function. The C terminus of this multidomain tetraspanning structure interacts with cytoplasmatic adaptor proteins. Consequently, it mediates signalling functions, as it regulates intercellular trafficking and facilitates occludin dimerization. Expression of occludin in the endometrium correlates with altered permeability of different segments in the vascular tree (Bazzoni & Dejana 2004; Cummins 2012). Further, matrix metalloproteinases (MMPs) mediates proteolytic cleavage of occludin. These result in a disruption of barrier function and increased permeability. Another aspect of barrier regulation is the posttranslational modification of junctional proteins. Therefore, occludin was identified

to serves as substrate of cellular kinases and phosphatases, e.g. phosphorylation of tyrosine or serine residues results in dysfunction of vessel barriers (Cummins 2012). Seebach et al supports the hypothesis that phosphorylation of occludin under flow conditions, leads to an increase of hydraulic conductivity (or decrease of resistance) (Seebach et al. 2007).

While all functions of occludin are still not completely investigated, it is mentioned that mutations or inactivation of members of the claudin-family (21-35 kDa), as compartments of TJs, causes diseases. For example, mutation of claudin-16 causes hypomagnesaemia, claudin-14 mutation induces deafness and absence of central nervous system myelin results in response to a claudin-11 mutation. Around 27 members belong to the claudin family, depending on the specific organ or tissue. All members consist of four membrane-spanning regions, whereas both, the long C and short N terminus, are oriented in the cytosol. So, the structure seems similar to occludin, but there is no sequence similarity between these two groups (Bazzoni & Dejana 2004). In general, the main function of this family is the paracellular sealing, which is tissue-, size- and charge-selective. In this case, sealing claudins, such as claudin-4, -5, -8, -11, -14 and -19 perform a selectively decrease of paracellular permeability. In contrast, claudins-2, -7, -10, -15, and -16 are known to have pore-forming function. Claudin-5 is expressed primarily in vascular endothelial cells, but claudin-1, -3, -7 and -12 are also found. The former binds homophilic, interacts calcium independently, has cell tightening function and is activated by protein kinase A. Its phosphorylation leads to a translocation from the cell membrane, resulting in increased paracellular permeability, destabilizing TJ-complexes. The tightening function of claudin-5 is necessary to reduce paracellular permeability for cations (Krause et al. 2008).

TJP1, TJP2 and TJP3 are essential for organisation of claudin-based strands. Furthermore they are essential in forming very stable complexes with transmembrane proteins at the edge of intercellular spaces in epithelial and endothelial cells. These three proteins belong to the protein family of membrane associated guanylate kinases (MAGUK), with the properties to link molecular complexes, e.g. synapses and TJs, and act as signal transduction molecules. This study mainly focuses on TJP1, but further information on MAGUK proteins is available in the review of González-Mariscal. TJP1 (~225kDa) belongs to peripheral membrane proteins that are components of TJs. However, these proteins are most frequent in epithelial and endothelial cells, but are also found in Schwann cells, fibroblasts, astrocytes, sarcoma and myeloma cell lines. In these, TJP1 is localized at the cytoplasm membrane to regulate

paracellular permeability, and on the nucleus, where it stimulates cell cycle progression and transcription. Consequently, expression of TJP1 plays an important role in flow resistance and permeability of the cell monolayer as a variety of agents, e.g. glucocorticoids and endothelial growth factors interact with this protein (González-Mariscal et al. 2000).

1.5. Diabetes in pregnancy

At present, three major types of diabetes are known, diabetes mellitus type 1 (DM1), type 2 (DM2) and gestational diabetes mellitus (GDM). While DM1 is classically caused by an autoimmune destruction of β-cells which are located in the pancreas, DM2 results from insulin resistance (Triplitt et al. 2015). In contrast to the other types of diabetes, GDM is described as a glucose intolerance that clinically manifests first only in pregnancy. Whilst there is a physiological insulin resistance occurring during second trimester in normal pregnancy, the insulin resistance in GDM results from an additional chronic insulin resistance (Buchanan & Xiang 2005). This results in hyperglycaemia and hyperinsulinemia in the fetal circulation. GDM causes morphological changes in the placenta, i.e. hypervascularisation as a result of increased surface area and length of capillaries, accelerated microangiopathy and changes in capillary permeability of feto-placental endothelium (Babawale et al. 2000). The observed alterations in maternal metabolism also results in consequences for the offspring, e.g. fetal macrosomia (Lao et al. 1996), increased risk for perinatal mortality, later obesity, DM2 and metabolic and cardiovascular disease, later in life (Gui et al. 2015). A decreased expression of adherens junctional molecules VE-cadherin and β-catenin as well as of the tight junctions expression of occludin and TJP1 was also observed in GDM. Hyperglycaemia and hyperinsulinemia contribute to these changes (Lassance et al. 2013).

But there is another factor that has an enormous influence on the placental and consequently the fetal development. During the first trimester of pregnancy the oxygen tension remains low but increases at the end of the first trimester (Jauniaux et al. 2000). Therefore, hypoxia is a key factor in inducing placental development, as it affects expression of pro-angiogenic factors that stimulate angiogenesis. Maternal hyperglycaemic and hyperinsulinaemic environment stimulates fetal metabolism that results in an increased oxygen demand. Thus, an inadequate oxygen supply may result in elevated expression of growth factors that will further affect vasculogenesis and angiogenesis. Stimulated angiogenesis in increased branching and enlargement of capillary surface area (Cvitic et al. 2014).

2. Hypothesis and objectives

Diabetes in adulthood is associated with endothelial dysfunction. Also diabetic environment in GDM may alter the function of placental vessels.

The aim of the study was to investigate whether GDM alters barrier function of feto-placental ECs. Therefore, AEC and VEC from placentas of female infants of normal and GDM pregnancies were analysed. Expression of TJ and AJ protein was quantified by real time PCR and Western blot, and barrier function was evaluated using ECIS. Furthermore, both, the current and permanent effect of hyperglycaemia on barrier function of ECs were tested. To this end, impedance of EC was measured during and after a three-day hyperglycaemic treatment.

3. Material and Methods

3.1. Isolation and cultivation of human feto-placental endothelial cells

Primary feto-placental AEC and VEC from third trimester placentas were isolated. Therefore, arterial and venous vessels with a length of approximately 2-4 cm were cut out of the chorionic plate and washed with Hanks' balanced salt solution (HBSS, Gibco). After cannula insertion (Optiva 2 I.V. Catheter Radiopaque, Smiths Medical) vessels were perfused with Collagenase/Dispase solution (1 mg/mL, Roche) solubilized in HBSS. Cells were collected in fetal calf serum (FCS, Hyclone) and centrifuged at 4 °C for 7 minutes at 900 rpm. The pellet was resuspended in Endothelial Basal Medium (EBM, Lonza) supplemented with EGM-MV BulletKit (Lonza) containing gentamicin/amphotericin, hydrocortisone, recombinant-human epidermal growth factor, bovine brain extract and 5 % FCS and cultivated on 1 % (v/v) gelatine-coated (Life Technologies) flasks (25 cm^2, 50 cm^2, 75 cm^2, Nunc).

Quality and purity of the cells was determined by indirect immunofluorescence staining. Then cells were grown at different conditions to mimic the oxygen variation in the placenta. Therefore, AEC were cultivated at 12 % O_2 and 37 °C and VEC at 21 % O_2 and 37 °C.

3.2. Electrical cell-substrate impedance sensing

Differences in permeability between ECs and GDM ECs were determined using the electrical cell-substrate impedance sensing system (ECIS, Applied Biophysics). Therefore, VEC (n=5) and GDM VECs (n=3) with a cell number of $1.1*10^5$ cells per well (400 µL of EBM) were plated on gelatine-coated gold electrodes (8W10E+ arrays, Applied Biophysics). AEC (n=6) and GDM AEC (n=9) needs a cell number of $8*10^4$ cells per well (400 µL of EBM) to plate out on gelatine-coated gold electrodes (8W10E+ arrays, Applied Biophysics). The cells were grown to confluency for 12 hours to adhere onto the electrodes. Then baseline of impedance was recorded at 4 kHz for 24 hours.

3.3. Real Time PCR

The total cellular RNA (of 6 GDM AEC, 6 AEC, 6 GDM VEC and 6 VEC) was isolated using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen). Quality and concentration of the RNA was measured by Scan Drop Spectrometer (Analytik Jena) using 260/280 ratio.

Total RNA (2 µg) was reversely transcribed to cDNA (40 µL) according to the manual SuperScript II Reverse Transcriptase (Invitrogen), using the C1000 Thermal Cycler (Biorad). 8.8 µL of cDNA was taken for real-time PCR (RT PCR) using FAM labelled TaqMan for the gene expression assay. Four gene specific primers of adherens junctions, *cadherin-5* (CDH5,

VE-cadherin, Hs00901463_m1, Life Technologies), *protocadherin1* (PCDH1, *γ-cadhenin*, Hs00170174_m1, Life Technologies), *catenin α 1* (CTNNA1, Hs00944794_m1, Life Technologies), *catenin β 1* (CTNNB1, Hs00355049_m1, Life Technologies) and seven primers responsible for tight junctions, *occludin* (OCLN, Hs00170162_m1, Life Technologies), *claudin-12* (CLDN12, Hs00273258_s1, Life Technologies), *claudin-7* (CLDN7, Hs00600772_m1, Life Technologies), *claudin-1* (CLDN1, Hs00221623_m1, Life Technologies), *claudin domain containing 1* (CLDND1, Hs00219886_m1, Life Technologies), *tight junction protein1* (TJP1, Hs01551861_m1, Life Technologies), *tight junction protein2* (TJP2, Hs00910543_m1, Life Technologies) were synthesized via TaqMan Universal PCR MasterMix (Applied Biosystems). Expression level of housekeeping gene ribosomal protein L30 (RPL30, Hs00265497_m1, Life Technologies) was used as an internal control because its expression was not influenced by gestational diabetes or the fetal sex. According to the manufactures instructions, the components were mixed and amplified in a total volume of 40 µL/well (Low-Profile 48-Well multiplate, BioRad). PCR was performed at the Core-Facility Molecular Biology Center of the Medical University of Graz using the Bio-Rad CFX96™ Real-Time PCR Detection System (Biorad). Thermal cycling conditions were: 10 min at 95 °C, followed by 40 cycles of alternated denaturation- (95°C for 15sec) and annealing/extension-phases (60°C for 1 min).

The threshold cycle values were defined automatically from the Bio-Rad CFX Manager software (BioRad). Copy numbers were determined according to the $2^{-\Delta\Delta Ct}$ method using ribosomal protein L30 as a reference. The reactions were performed in duplicates.

3.4. Western blot

Protein expression level of junctional structures was determined, after feto-placental ECs were grown to confluency in 75 cm^2-flasks (Nunc). Protein was extracted (10 µg/µL) from 6 AEC, 6 VEC, 5 GDM AEC and 6 GDM VEC, following the manual radioimmunoprecipitation assay buffer (RIPA buffer, Sigma Aldrich). Therefore, cells were washed with HBSS. Then 300 µL of icecold RIPA buffer with proteinase inhibitor was added. After cells were scratched from the flask, they were incubated on ice for 30 minutes. To generate a high protein concentration, samples were treated with ultrasound twice for 10 sec with intermittent incubation on ice for 30 sec. Then a centrifugation step at 13 000 rpm at 4 °C for 20 minutes was performed. The pellet was discard and the concentration of protein was determined using bicinchoninic acid (BCA) assay. According to the protein concentration, lysates were mixed with Laemmli buffer (2x, Sigma Aldrich) and denatured at

95 °C for 5-10 minutes. Samples (10 µg protein/well) were loaded onto a 4-20 % SDS-PAGE gel (BioRad) and run at 110 V with 1x Tris/Glycine/SDS running buffer (BioRad) for 1 hour. As a standard Page Ruler plus Prestained Protein Ladder (#26619, Thermo Scientific) was used. Proteins were transferred onto nitrocellulose membrane (BioRad) at 1.3 A (25 V) for 10 min. The total protein was visualized after incubation of the membrane for 5 minutes in staining solution (0.1 % (w/v) Ponceau S in 5 % (v/v) acetic acid, Sigma Aldrich). Stain was removed from protein bands by washing with distilled water. Nonspecific binding sites were blocked for 1 hour with 5 % non-fat dry milk (BioRad) in 0.1 % Tween 20 (Sigma Aldrich) in tris-borate-EDTA (TBE) or in 2 % bovine serum albumin (BSA) in TBE at room temperature. Primary antibodies against occludin (ab31721, abcam), VE-cadherin (#2158, CellSignaling), claudin-5 (ab53765, abcam) and paxillin (ab32084, abcam) were used at dilution of 1:1000 and tight junction protein 1 (AA 1048-1247, antibodies-online) at 1:2000. The membranes were incubated with primary antibodies overnight at 4 °C. Then membranes were washed five times with TBE and incubated with the appropriate secondary antibody (goat anti mouse or anti rabbit, 1:2000, BioRad Laboratories) conjugated with horseradish peroxidase. Then membranes were visualized using the enhanced SuperSignal™ West Pico chemiluminescent detection system (Thermo Scientific) and Chemidoc XRS software (BioRad). Protein bands were visualized using Alpha Digidoc software (Alpha Innotech Corp).

3.5. Glucose memory effect

The effect of long term hyperglycaemia on barrier function was determined. Therefore, three impedance measurements for normal VECs (n=4) and GDM-derived VECs (n=3) were performed, using the ECIS system (Applied Biophysics) (Fig. 1). Cells were grown with EBM (5.5 mM D-glucose) on electrodes and then impedance was measured using the settings previously described (1). After a hyperglycaemic (15 mM D-glucose) three-day-treatment, impedance was measured again. As a reference, VECs and GDM VECs were cultured normoglycaemic (5.5 mM D-glucose) with additional treatment with 9.5 mM L-glucose (2). To identify how long the effect of altered barrier function lasts, another ECIS experiment was performed after cells were grown in normoglycaemic EBM (5.5 mM D-glucose) for 3 days (3).

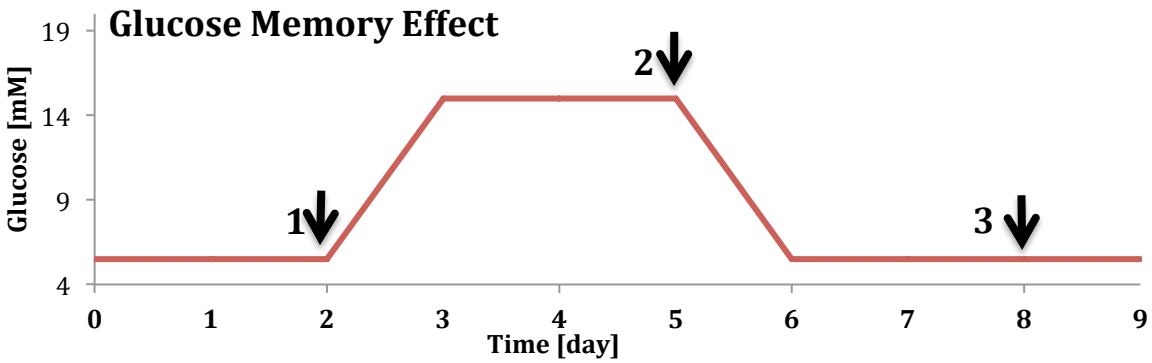

Fig. 1: Schematic depiction of impedance measurement to determine the glucose memory effect of ECs. First measurement was taken when cells were grown to its confluence (1). After treatment with 15 mM D- and L-glucose impedance was measured to define differences in cell permeability (2). Third measurement was done when cells were grown again with normal glucose concentration (5.5 mM) for three days.

3.6. Statistical analysis

Differences between normal and GDM derived ECs, as well as between AEC and VEC were analysed. Results from real time PCR were analysed according to the $2^{-\Delta\Delta Ct}$ method using the gene encoding ribosomal protein L30 as a reference. Statistical analyses of impedance measurements and Western blot analyses were performed using student t-test (paired samples). Significance was accepted at values of $p<0.05$ and $p<0.01$. Statistical analyses were performed with Prism 6 software (GraphPad).

4. Results

To test alterations in barrier function and permeability of ECs, impedance measurements of AECs, VECs isolated after normal and GDM complicated pregnancy were performed (Fig. 2-4). Gene expression level of tight and adherent junctional proteins was determined via RT-qPCR. Consequently, these results were verified via Western blot. The last part of the study concerns the hypothesis, that an increase in D-glucose level affects feto-placental endothelial barrier function. To this end, ECs were treated with 15 mM D-glucose and changes in permeability were determined by impedance measurement using the ECIS system.

4.1. Electrical cell-substrate impedance sensing

Differences in the permeability between ECs and GDM-derived ECs were determined using the ECIS method. Alterations in permeability between AECs (n=6) and VECs (n=5) were specified as it is shown in figure 2. Therefore impedance baseline was recorded for 24 hours. It can be seen, that AECs revealed slightly higher impedance than VEC. This increase remains steady during the first 12 hours of the assay, but differences did not reach significance.

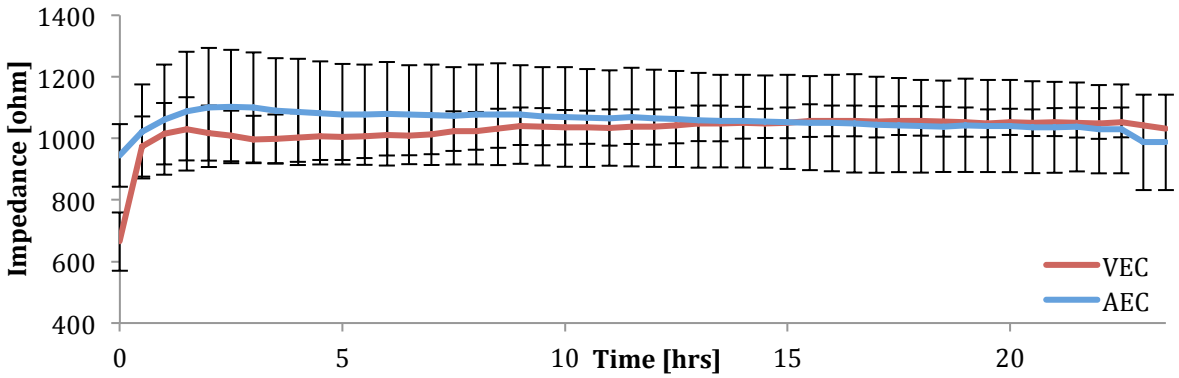

Fig. 2: Impedance measurement of AECs (red) compared with VECs (blue). Baseline was measured for 12 hours with an average impedance of 1000 ohm for VECs (n=5) and 1100 ohm for AECs (n=6).

Then, changes in barrier function between AECs and GDM AECs were determined (Fig. 3). To this end, impedance of AECs (n=6) and GDM-derived AECs (n=9) was measured for 24 hours. The graph, as seen in Fig. 3, indicates higher impedance and consequently lower permeability within the GDM AEC group that remains steady from the 1st till 20th hour (+17.8%; $p<0.05$).

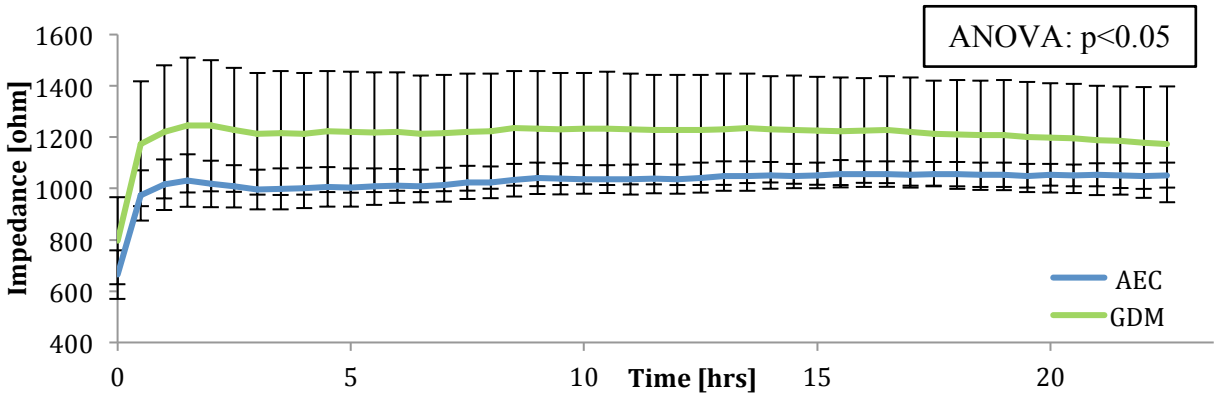

Fig. 3: Impedance measurement of AECs (blue) compared with GDM AECs (green). Impedance baseline was recorded for 24 hours with an average impedance of 1026 ohm for AECs (n=6) and 1209 ohm for GDM AECs (n=9). Statistical analyses determined significant differences between the two groups during the 1st till the 20th hour (p<0.05).

When comparing VEC after normal (n=5) vs. VEC after GDM pregnancy (n=3), reduced impedance was identified. The graph shows significant lower impedance in GDM VEC that remains steady during the 7th and 24th hour (-23.4%; p<0.05) (Figure 4).

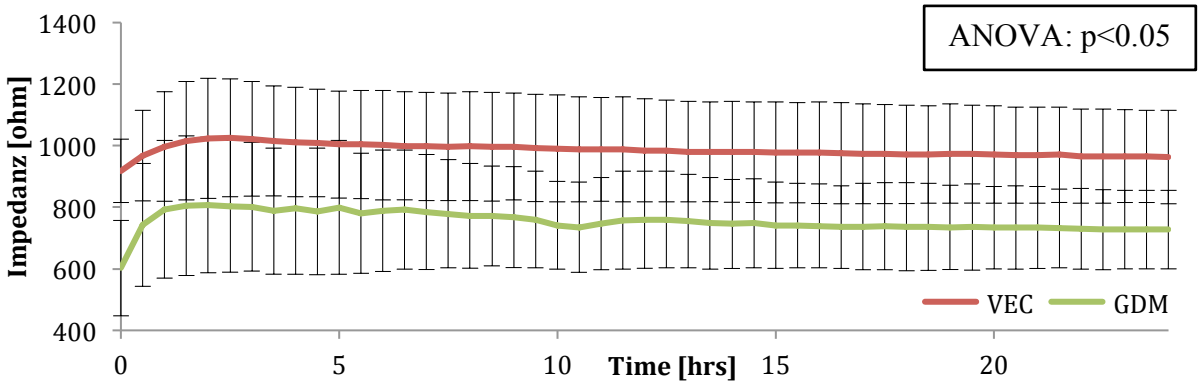

Fig. 4: Impedance measurement of VECs (red) compared with GDM VECs (green). Baseline was measured for 24 hours with an average impedance of 985 ohm for VECs (n=5) and 754 ohm for GDM VECs (n=3). Statistical analyses showed significant results during the 7th till the 24th hour (p<0.05).

4.2. Real Time-PCR

Differences in mRNA expression of junctional proteins were determined performing real time PCR. Three genes encoding AJ proteins and seven genes encoding TJ proteins were measured. Of the 10 surveyed genes, 2 were differentially expressed in GDM derived cells. In Table 1 all expression differences of the surveyed genes (Tab. 1), expressed in feto-placental ECs of female infants are observed. To determine alterations in expression of TJs and AJs, RT-qPCR of VE-cadherin, α and β catenin from AJs and occludin, claudin domain containing protein 1, claudin-1, claudin-7, claudin-12 and tight junction protein 1 and 2 as member of TJs was performed.

Tab. 1: mRNA expression of AJ (CDH5, CTNNA1 and CTNNB1) and TJ proteins in AECs, VECs and GDM counterparts of female infants was determined. CDH5 and TJP1 showed deceased expression level in VECs (n=3) compared to AEC (n=3), whereas expression of OCLN was increased. CTNNA1 was increased in GDM AECs (n=3), compared to AECs. In GDM VEC (n=3), CLDN7 was decreased compared to VECs. Expression of CLDND1, CLDN1, CLDN12, CTNNB1 and TJP2 was not influenced by vessel type or diabetic disease.

		AEC vs. VEC		AEC vs. GDM AEC		VEC vs. GDM VEC	
Gene	**Symbol**	**fold change**	**p-value**	**fold change**	**p-value**	**fold change**	**p-value**
VE-cadherin	CDH5	-0.86	**<0.05**	1.40	0.53	-0.32	0.47
***α* catenin 1**	CTNNA1	-0.97	0.67	1.81	**0.02**	-0.14	0.19
***β* catenin 1**	CTNNB1	-0.59	0.14	1.09	0.93	-0.36	0.34
occludin	OCLN	1.20	**<0.01**	1.44	0.94	-0.34	0.06
claudin domain containing protein 1	CLDND1	-0.76	0.86	1.40	0.36	-0.32	0.05
claudin-1	CLDN1	-0.66	0.32	-0.71	0.40	-0.72	0.99
claudin-7	CLDN7	-0.61	0.97	-0.83	0.30	-0.37	**0.02**
claudin-12	CLDN12	-0.22	0.13	-0.81	0.73	-0.53	0.11
tight junction protein 1	TJP1	-0.80	**0.04**	1.25	0.61	-0.24	0.35
tight junction protein 2	TJP2	1.04	0.61	2.46	0.08	-0.14	0.05

Comparison of AECs (n=3) with VECs (n=3) showed decreased expression of VE-cadherin (-14%; $p<0.05$) and tight junction protein 1 (-20%; $p=0.04$) whereas mRNA level of occludin (+20%; $p<0.01$) was increased in VEC (Table 1). Results observed in diabetic AECs and VECs, compared to ECs of normal pregnancies are illustrated in Fig. 5. The expression of α-catenin (+81%; $p=0.02$) is increased in GDM AECs (n=4) compared to AECs. In GDM VECs (n=3) mRNA expression of claudin-7 (-55%; $p=0.02$) declines compared to VECs.

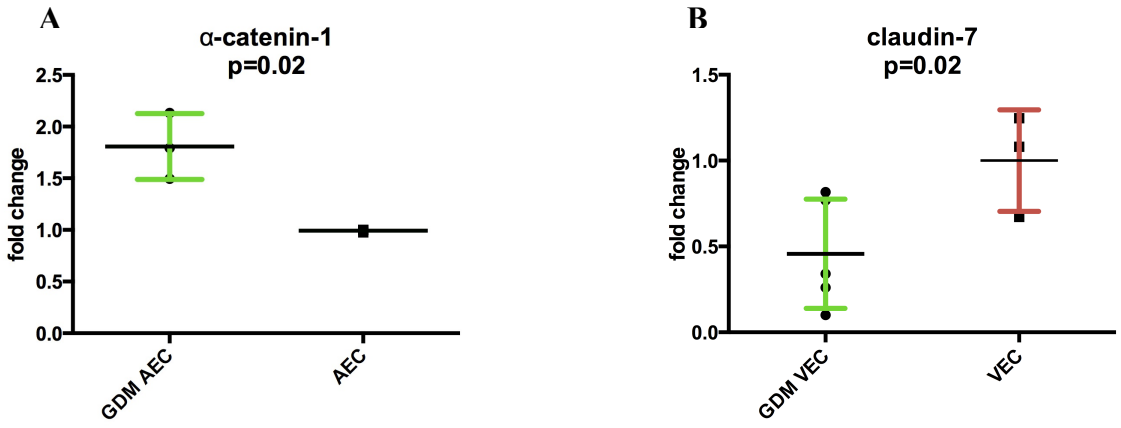

Fig. 5: Graph A shows increased expression level of α-catenin-1 in GDM AEC (green; n=4; p=0.02) compared to AECs (black; n=3). Decreased mRNA expression was observed in claudin-7 in GDM VEC (green; n=3) compared to VECs (red; n=3) (graph B).

Comparison of normal (n=6) and diabetic AECs (n=6) regardless of the fetal sex revealed increased expression of claudin domain containing 1 (CLDND1; +78%; $p<0.01$), VE-

cadherin (CDH5; +54%; p<0.05), tight junction protein 1 (TJP1; +61%; p<0.05) and α-catenin (CTNNA1; + 85%; p<0.01) (Fig. 6) in AEC GDM. There was an increase of all four junctional proteins in GDM AECs whilst there was difference in junctional proteins in VECs (n=6) compared to GDM VECs (n=6).

Genes whose expression reached significance level of p<0.05 in students t-test, were taken for Western blot analyses.

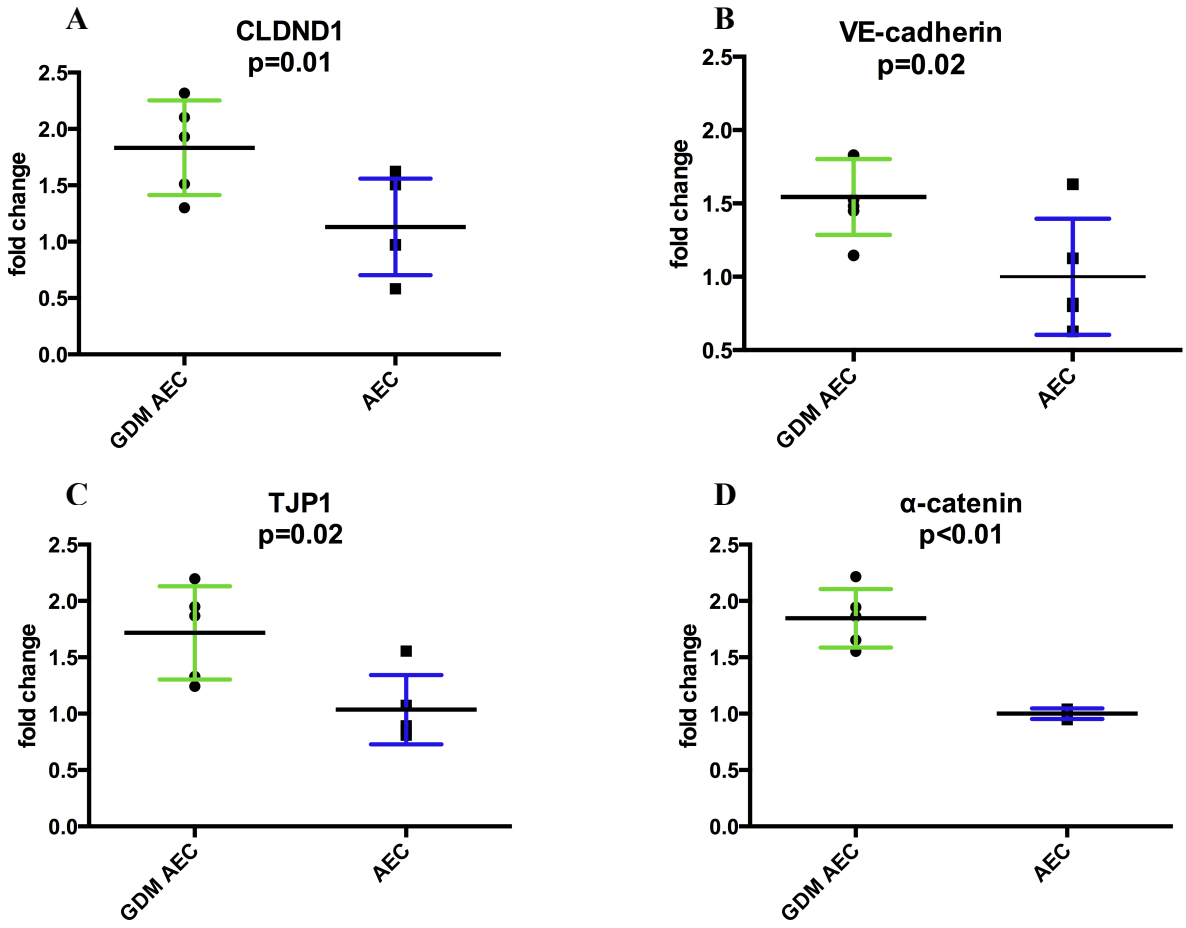

Fig. 6: CLDND1, CDH5, TJP1 and α-catenin mRNA expression in GDM AEC (n=6, green). Increased expression level of CDH5 (p=0.02), CLDND1 (p=0.01), TJP1 (p=0.02) and α-catenin (p<0.01) was observed in GDM AEC. Fold change of protein expression from GDM AEC has been normalized to AECs (control, n=6, blue).

4.3. Western blot

To confirm the RT-qPCR results on protein level Western blot experiments were performed. Therefore, proteins from confluently grown ECs (VECs, AECs and GDM) were isolated. Antibodies against junctional proteins, that showed increased fold change on mRNA levels, and furthermore, the expression of two focal adhesion proteins, vinculin and paxillin, were determined.

First AECs (n=6) were compared with VECs (n=4). The results show that the expression level of β-catenin (+320%; $p<0.05$) and VE-cadherin (+305%; $p<0.05$) is higher in AECs (Fig. 7, Fig. 8). Both junction proteins are main components of AJ.

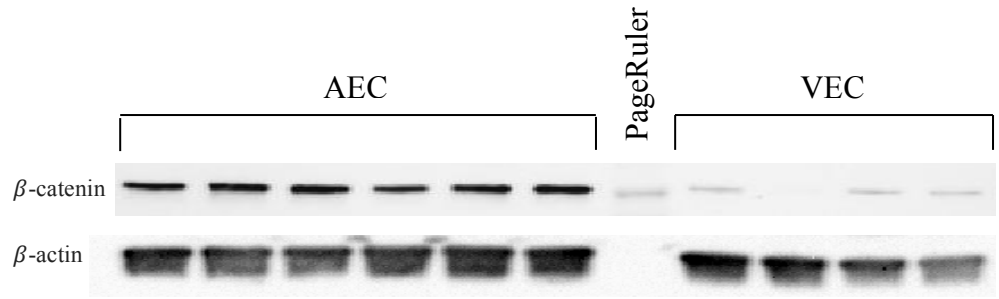

Fig. 7: Expression of β-catenin (94kDa) in AEC (n=6) and VEC (n=4). As internal control expression of β-actin (40 kDa) was used. In AECs protein expression of β-catenin is increased compared to VECs ($p<0.05$).

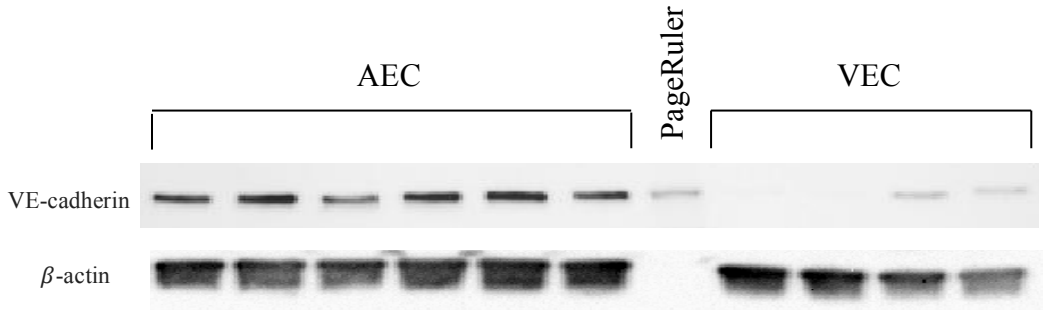

Fig. 8: Expression of VE-cadherin (130-140 kDa) in AEC (n=6) and VEC (n=4). As internal control expression of β-actin (40 kDa) was used. In AECs protein expression of VE-cadherin is increased compared to VECs ($p<0.05$).

Paxillin (+120%; $p<0.05$) was higher in AECs compared to VECs (Fig. 9). The comparison of vinculin, as second focal adhesion protein, is not shown, as its difference in protein expression did not reach significance.

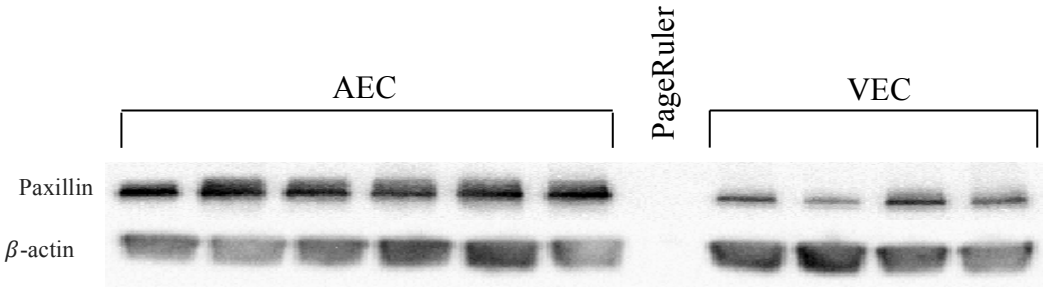

Fig. 9: Expression of paxillin (60 kDa) in AEC (n=6) and VEC (n=4). Expression of β-actin (40 kDa) was determined as internal control. In AECs expression level of paxillin is increased, compared to VECs ($p<0.05$).

In AECs only TJP1 (+350%; $p<0.05$) had higher protein levels when compared to VECs (Fig. 10).

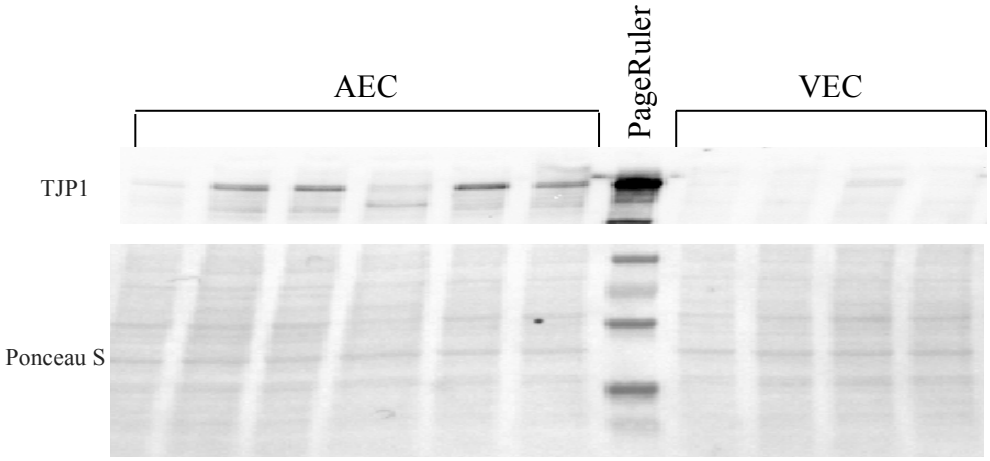

Fig. 10: Expression of TJP1 (220 kDa) in AEC (n=6) and VEC (n=4). As internal control the total protein expression, labelled via ponceau S staining, was used. In AECs, expression of TJP1 is increased, compared to VECs ($p<0.05$).

Then, differences in protein expression between GDM-derived AEC and VEC vs. normal AEC and VEC were determined. Protein data in AECs (n=6) confirmed an increase in expression level of TJP1 (+181%; p=0.008) in GDM AEC (n=5) (Fig. 11), whereas no significantly different level was found for the other junctional proteins. To compare the results with the VECs, one primary isolation of AECs was loaded onto the membrane.

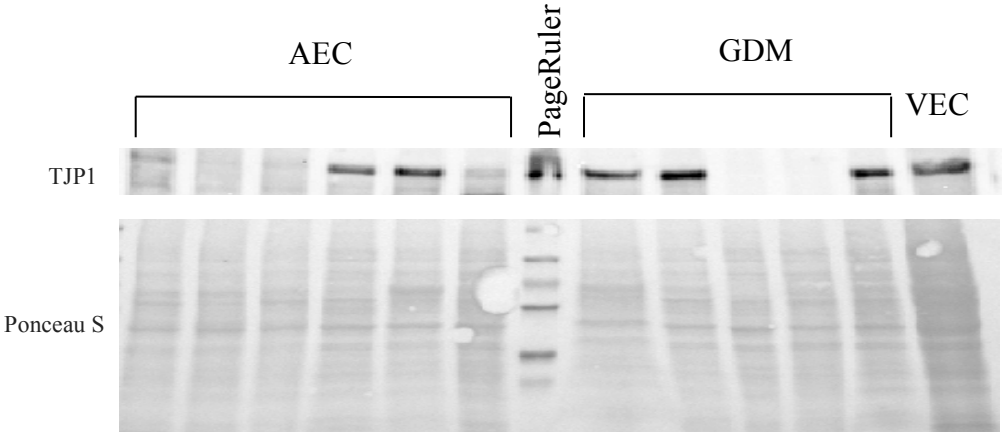

Fig. 11: Expression of TJP1 (220 kDa) in AEC (n=6) and GDM AEC (n=5). As internal control the total protein expression, labelled via ponceau S staining, was used. TJP1 expression was increased in GDM AECs compared to AECs ($p<0.01$).

To determine alterations in protein expression between control and GDM derived VEC, six different VEC and GDM VEC isolations were chosen. Protein expression of VE-cadherin (-51%; p=0.024) was decreased in GDM derived VEC, whereas claudin-5 (+33.6%; p=0.018) level was increased in GDM VECs compared to the control group (Fig. 12, Fig. 13).

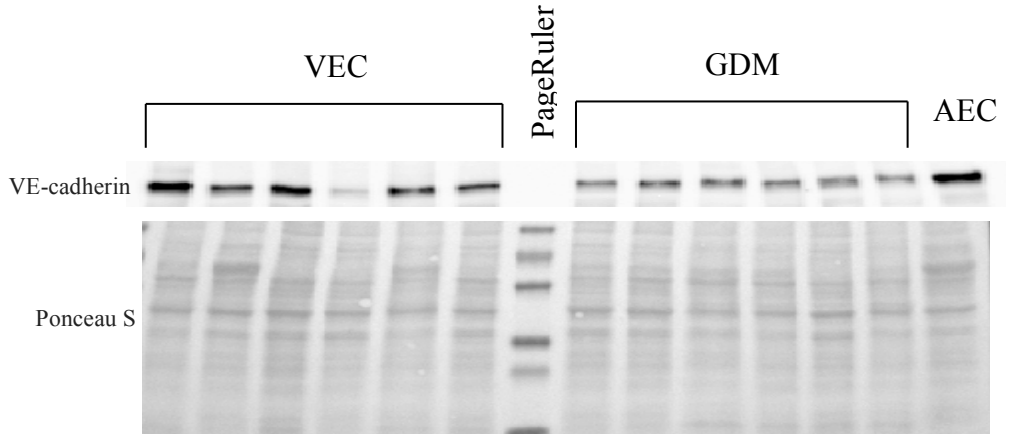

Fig. 12: Expression of VE-cadherin (115 kDa) in VEC (n=6) and GDM VEC (n=6). As internal control total protein expression, labeled via ponceau S staining, was determined. VE-cadherin expression was decreased in GDM VECs compared to the control group (p<0.05).

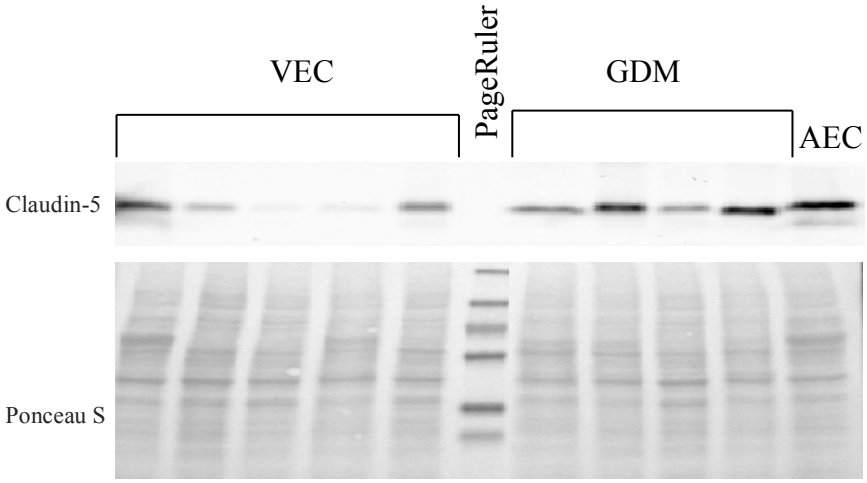

Fig. 13: Expression of claudin-5 (23 kDa) in VEC and GDM VEC. As loading control, total proteinwas stained with Ponceau S Claudin-5 expression was increased in GDM VECs compared to the control group (p<0.02).

All results from protein analysis of AECs compared with VECs are shown in Tab. 2. VE-cadherin (+305%; p<0.01), β-catenin (+320%; p<0.01), TJP1 (+350%; p<0.01) and paxillin (+120%; p<0.05) were higher expressed in AEC than in VEC, whereas expression of occludin and vinculin remained steady.

Tab. 2: Protein expression in AECs compared to VECs.

	Gene Symbol	AEC	VEC	p-value
β-catenin	CTNNB1	36	8	**<0,01**
VE-cadherin	CDH5	36	9	**<0,01**
tight junction protein 1	TJP1; ZO-1	43	3	**<0,01**
paxillin	PXN	193	88	**<0,05**

occludin	OCLN	16	28	0,28
vinculin	VCL	65	66	0,98

In VEC, GDM decreased protein expression of VE-cadherin (-51%; $p<0.05$) and increased protein level of claudin-5 (+336%; $p<0.05$) was observed. Protein production of TJP1 and occludin remained unchanged (Tab. 3).

Tab. 3: Comparison of protein expression between VECs and GDM VECs.

	Gene Symbol	VEC	GDM VEC	p-value
claudin-5	CLDN5	19	56	**<0,05**
VE-cadherin	CDH5	58	29	**<0,05**
tight junction protein 1	TJP1; ZO-1	16	8	0,77
occludin	OCLN	5	22	0,10

Comparison of GDM AECs with the control group after normal pregnancies demonstrated reduced protein expression level of TJP1 (-44%; $p<0.01$) in AECs. Protein expression of claudin-5 and occludin displayed no difference (Tab. 4).

Tab. 4: Protein expression of AECs compared to GDM AECs.

	Gene Symbol	AEC	GDM AEC	p-value
claudin-5	CLDN	75	54	0,44
tight junction protein 1	TJP1; ZO-1	13	36	**<0,01**
occludin	OCLN	15	14	0,92

4.4. Glucose Memory Effect

The long-lasting affect of a hyperglycaemic environment was tested on VECs (n=4). Therefore series of impedance measurements with cells treated with increased D-glucose level were performed. As a reference value, average impedance baseline, which is 1100 ohm, was taken (Fig. 14).

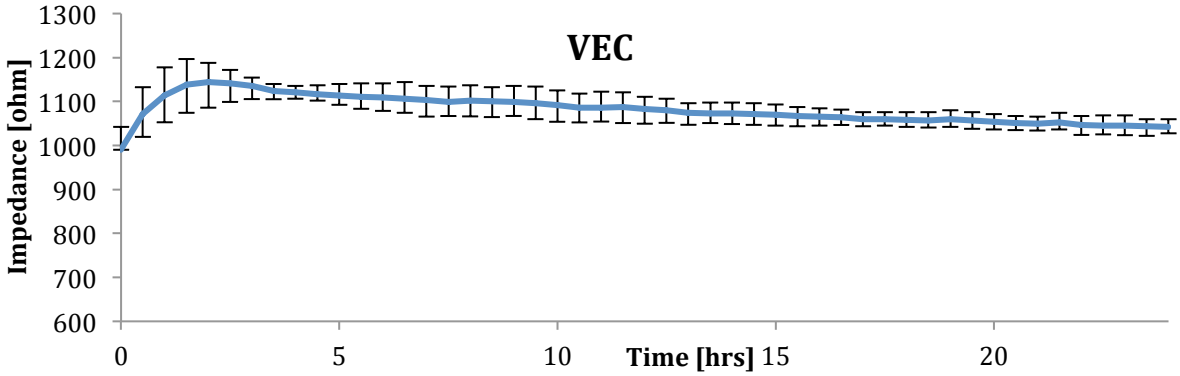

Fig. 14: Impedance of VECs (n=4) was measured for 24 hours. Average impedance of VECs was about 1100 ohm.

The cells were treated with 15 mM D-glucose for three days after which period the impedance measurement was repeated. L-glucose was used as an osmotic control since changes in osmotic pressure can affect cells as well. L-glucose cannot be transported into the cells and hence, cannot be metabolized. VEC (n=3), treated with D-glucose tended to form a better barrier when compared to the control group treated with L-glucose. Generally, decreased impedance was detected after the hyperglycaemic environment (Fig. 15).

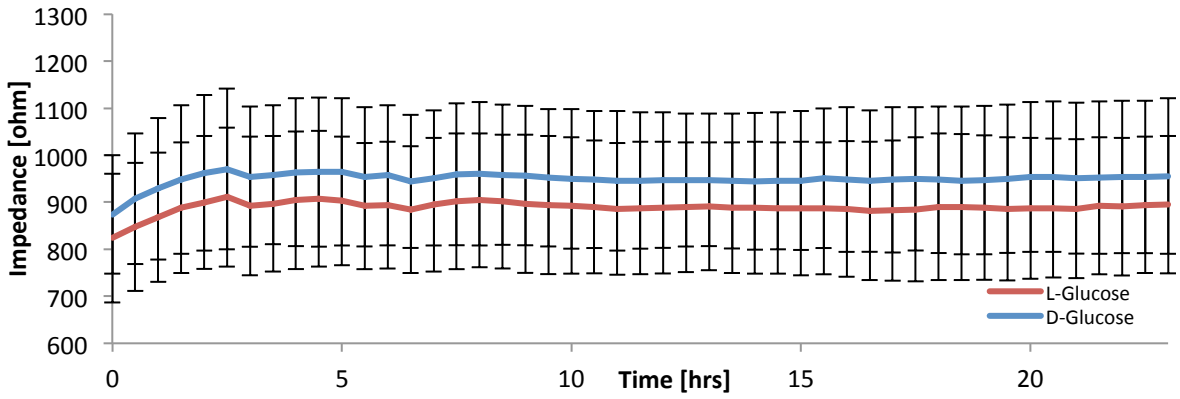

Fig. 15: Impedance of VECs (n=3) was measured for 24 hours. VECs (n=3) were treated with 15 mM D- and L-glucose, respectively. Average impedance of VECs treated with D-glucose (blue) was about 950 ohm and with L-glucose (red) 880 ohm.

After treatment with high glucose, cells were cultured under normoglycaemia (5.5 mM D-glucose) again for three days. Then, VEC (n=3) monolayer impedance was measured again to detect whether hyperglycaemia affect cells in long term. The impedance of cells treated with L-glucose was elevated, whereas in VECs treated with D-glucose barrier function remains unchanged (Fig. 16).

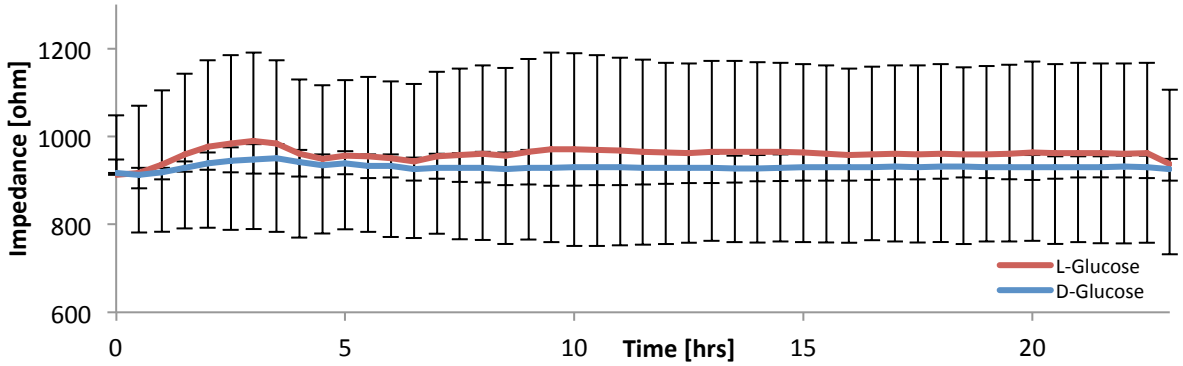

Fig. 16: Impedance of VECs (n=3) was measured for 24 hours. After switching to normoglycaemic conditions for another three days impedance was measured again. Average impedance of VECs treated with D-glucose (blue) and L-glucose (red) was 940 ohm.

The effect of a hyperglycaemic environment was also tested on GDM derived VEC. Impedance of confluent GDM VEC (n=3) was measured. As a reference value an average impedance baseline of 650 ohm was recorded (Fig. 17).

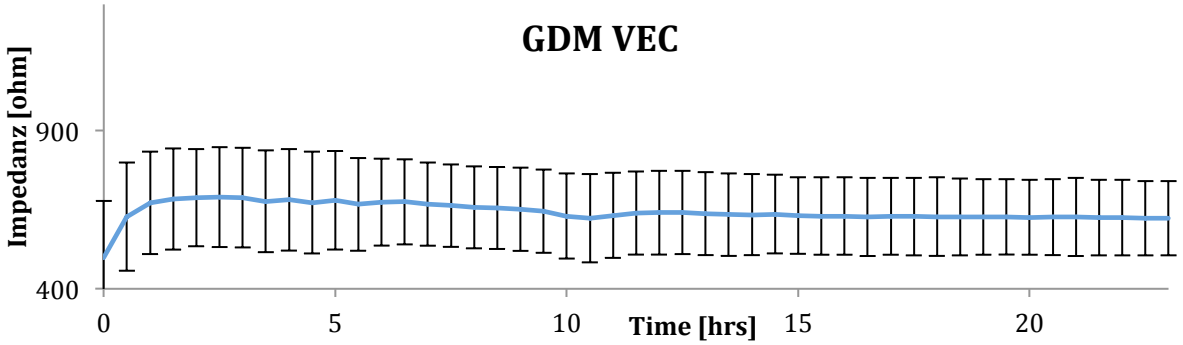

Fig. 17: Impedance of GDM derived VECs (n=3) was measured for 24 hours. Average impedance of GDM VECs was about 650 ohm.

As a next step, cells were treated with 15 mM D-glucose for three days after which time the impedance measurement was repeated. Again, L-glucose was used as control treatment. D-glucose treatment of GDM VEC (n=3) increased impedance to approximately 1050 ohm, but difference did not reach significance. Generally, hyperglycaemia induces increased impedance of cells (Fig. 18).

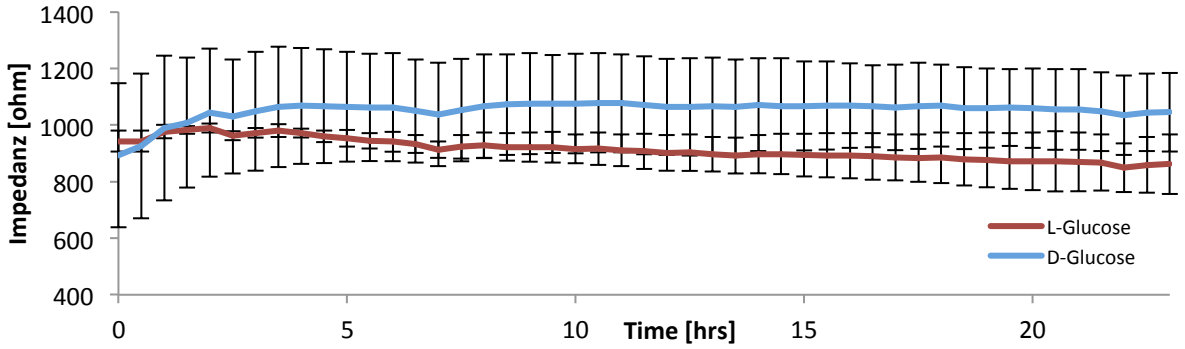

Fig. 18: Impedance of GDM derived VECs (n=3) was measured for 24 hours. GDM VECs were treated with 15 mM D- and L-glucose, respectively. Average impedance of GDM VECs treated with D-glucose (blue) was about 1050 ohm and with L-glucose (red) 910 ohm.

To determine whether hyperglycaemia causes long term effects, impedance was measured after three days at normoglycaemia again. GDM-derived VEC treated with high level of D-glucose had higher impedance than GDM-derived VEC treated with control between 8 and 24 hours of the measurement (Fig. 19).

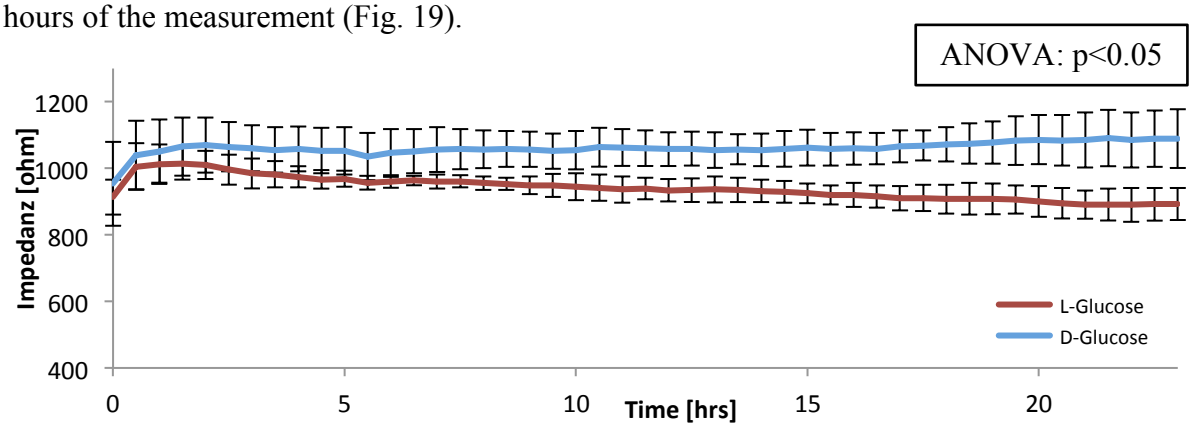

Fig. 19: Impedance of GDM derived VECs (n=3) was measured for 24 hours. After switching to normoglycaemic conditions for another three days, impedance of the monolayer was measured again. The average impedance of GDM VECs treated with D-glucose (blue) was 1060 ohm and L-glucose (red) 940 ohm. Statistical analyses showed significant results between the two groups from 8 to 24 hours of the measurement ($p<0.05$).

In general when one looks at the figures of the VEC, a slightly decrease in impedance level was observed when treated with glucose. But afterwards when the cells were cultured under normoglycaemia the impedance remains steady at the same low level.

From the figures of the GDM VECs it is apparent that there is a dramatically increase in impedance, detected after treatment with glucose. But then, the impedance of the cells remains steady at high level, regardless of hyperglycaemic or normoglycaemic environment.

5. Discussion

The results of the present study demonstrates that GDM alters monolayer resistance of feto-placental endothelial cells, possibly as a consequence of changes in AJ and TJ protein levels. According to the hypothesis that changes in the integrity of cell monolayer occurs in GDM, impedance measurements were performed. Referred to the ECIS measurements of AEC, it is clear that impedance level tends to be higher in AECs than in VEC during the first 12 hours. Consequently, the repertoire of junctional molecules was analysed to determine differences between AEC and VEC. At gene expression level, TJP1 and VE-cadherin were decreased, whereas occludin showed increased expression in VECs, compared to AECs. Performing Western blot analyses, AECs displayed increased protein expression of β-catenin, VE-cadherin, paxillin and TJP1. Thus, elevated impedance of AECs might occur as a consequence of increased expression of VE-cadherin and TJP1, which was verified in RT-PCR and Western blot analyses. Further, increased protein expression of β-catenin, as consequence of elevated VE-cadherin expression as it indicates increased occurrence of AJs, was determined. Therefore, association of VE-cadherin with catenins is required for cellular control of endothelial permeability and junction stabilization. A mutant form of VE-cadherin that was manipulated in binding to β-catenin or to plakoglobin, still forms intercellular zipper-like structures but the junctional strength is decreased. In β-catenin-null mouse embryos a failure of the cellular barrier of the fragile vessels was determined after exposure to elevated blood flow (increased blood pressure), resulting in haemorrhage (Dejana et al. 2008).
Additional, higher paxillin expression in AEC might indicate that AECs adhere faster to each other, built more stable cell-cell adhesion and consequently, grow much faster than VECs. Though, it requires further research to identify what kind of structures, might additionally influence cellular permeability and what important role they play in the molecular context.

Further experiments identified changes in permeability between AECs and GDM AECs. The study revealed that GDM increased AEC impedance. The improved barrier function was paralleled by elevated mRNA expression of α-catenin. Additionally, protein expression of TJP1 was also elevated. Elevated expression of α-catenin suggests that more AJs were formed, or actin bundling as a result of cell-reorganisation (Dejana et al. 2008). In early stages of adherens junctional development, TJP1 is expressed in ECs. Thus, it might be possible that TJP1 was detected because cell-monolayers were not completed (Bazzoni & Dejana 2004). Consequently other junctions were not formed and proteins thus, could not be detected by

Western blot analyses in these AEC. This suggestion is further confirmed as Babawale et al. confirmed that TJP1 was reduced in GDM (Babawale et al. 2000).

In VECs opposite results of GDM on barrier function were observed as impedance of GDM derived VECs was lower compared to normal VECs. GDM VEC further had lower mRNA expression of claudin domain-containing protein 1 (termed as claudin-25 in Mineta et al. 2011) and claudin-7. Protein expression of claudin-5 was increased in GDM VECs, whereas VE-cadherin was decreased. This leads to the hypothesis that VE-cadherin reduction contributes to the reduced cellular permeability of VECs in GDM. The mechanism that changes cellular permeability is very complex and might vary but it is known that VE-cadherin internalisation also reduces junctional strength (Dejana et al. 2008). Besides, it is indirectly involved in formation of TJs, as it stimulates claudin-5-expression by reduction of β-catenin translocation into the nucleus (Vestweber et al. 2008). Lower VE-cadherin levels may thus reduce claudin 5.

Diabetes mellitus is associated with an increase of vascular leakage or permeability. Hempel et al showed that elevated glucose levels (20 mmol/L) cause higher activity of intercellular signalling molecules, e.g. protein kinase C (PKC). Hyperglycaemic environment results in this case in glucose-induced translocation (activation) of PKCα and PKCε, in parallel with a dose-dependent increase in endothelial permeability (Hempel et al. 1997). Furthermore, cells that were exposed to high glucose level (25 mM) for 2 hours, show significant perturbation of junctional VE-cadherin in HUVECs (Payne & Leach 2000). The study from Liu et al showed that after treatment with high glucose, protein expression of TJP1, occludin and claudin-5 was reduced in the endothelial blood-brain barrier (Liu et al. 2012) but also in retinal endothelial cells (Tien et al. 2013).
Recent studies also confirmed that increased glucose concentration (25 mM) leads to a phosphorylation of VE-cadherin in HUVECs. As a result of this, the VE-cadherin- β-catenin complex, which is a main part of AJ, dissociates. This is followed by intracellular accumulation of β-catenin (as a result of phosphorylation of GDK3beta) and transactivation of β-catenin responsive genes which may contribute to vascular wall inflammation (Haidari et al. 2014).

Analysing the long-term effect of hyperglycaemia on the barrier function of normal and GDM VEC, impedance measurements of confluent grown cell monolayer was performed. The

experiment revealed elevated impedance level after three-day incubation with increased glucose concentration.

These results were unexpected as expression of claudin-7 and protein production of VE-cadherin, as main part of AJ, remains low in GDM VECs. Also a tendency in reduced expression of TJP2 and claudin domain containing protein 1 was observed in GDM VECs. These findings lead to the assumption of fewer junctional structures located in cell membranes or junctional disassemble that may result in a decline of impedance. Consequently this indirectly results in increased permeability. Previous studies, which were mentioned above, also confirm an opposite effect (concerning the result of this study) after glucose treatment.

It is known that impedance increases as response to shear stress, as it occurs under flow conditions in the vessel, followed by a gradual decline after 24 hours (Seebach et al. 2007).

To determine if the cells behave differently in another environmental and more natural context, e.g. under flow conditions combined with hyperglycaemia, further experiments need to be designed.

The present work showed that maternal GDM strongly affects barrier function of feto-placental endothelial cells. Thus, resulting in alterations of permeability as a possible consequence of junctional reorganisation in cellular membrane of the placental vasculature. However, the detailed changes in the maternal vasculature and the consequences for the fetus still remain unsure.

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