Diplomarbeit

Influence of diabetic conditions on the human placenta

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Ort, Datum …….. (Unterschrift)

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1 Zusammenfassung auf Deutsch

Hintergrund: Diabetes in der Schwangerschaft führt zu Pathologien der menschlichen Plazenta und somit auch des Feten. Ziel der Arbeit war es herauszufinden wie sich diabetische Bedingungen auf die Expression der MMPs und der enzymatischen Antioxidantien in der menschlichen Plazenta auswirken.

Material und Methoden: Humane Plazenta-Explants des ersten Trimenons wurden unter diabetischen Bedingungen, d.h. unterschiedlichen Glucose (16mM und 25mM) und Sauerstoff Konzentrationen (2,5%, 8%, 12% und 21%) inkubiert und hierauf die Genexpression von MMP2, 7, 9 und 19, sowie die enzymatischen Antioxidantien SOD3, NOX5 und GPX3 mittels RT-PCR untersucht.

Die statistische Auswertung erfolgte mittels SPSS fuer Windows unter Verwendung von Shapiro-Wilk-Test, Kolmogorov-Smirnof-Test (Lilleforts Variante), Mann-Lavene-Test, Mann-Whitney-U-Test, H-Test nach Kruskal und Wallis, t-Test und ONEWAY ANOVA.

Ergebnisse: Wir konnten keine signifikanten Veränderungen der Genexpression der untersuchten Gene bei unterschiedlichen Glucose- und Sauerstoffkonzentrationen feststellen. Lediglich gewisse Trends konnten bei einzelnen Genen angenommen werden.

Conclusio: Sowohl die MMPs als auch die antioxdativen Enzyme spielen bei der Implantation der Plazenta eine Rolle und werden durch diabetische Bedingungen beeinflusst, jedoch konnten wir dies in unserem Experiment nicht nachweisen.

2 Abstract

Background: Diabetes in pregnancy causes pathologies in the human placenta and the fetus. Aim of this work was to investigate the effect of diabetic conditions on the expression of the MMPs and the enzymatic antioxidants in the human placenta.

Material and Methods: Human placental explants of the first trimenon were cultivated under diabetic conditions, such as different glucose (16mM and 25mM) and oxygen concentrations (2.5%, 8%, 12% and 21%), and gene expression of MMP2, 7, 9 and 19 and the enzymatic antioxidants SOD3, NOX5 and GPX3 was subsequently analyzed via RT-PCR.

The statistical evaluation was performed by SPSS for windows by using of Shapiro-Wilk-Test, Kolmogorov-Smirnof-Test (variant of Lilleforts), Mann-Lavene-Test, Mann-Whitney-U-Test, H-Test of Kruskal and Wallis, t-Test and ONEWAY ANOVA.

Results: We could not find any significant changes in expression of the investigated genes under the different glucose and oxygen concentrations. Only certain trends in single genes may be assumed.

Conclusion: The MMPs as well as the antioxidative enzymes play a role in the implantation of the placenta and may be influenced by diabetic conditions; nevertheless our experiments could not prove this hypothesis.

3 Introduction

3.1 Development of the human placenta

The matutinal development of the fertilized egg, called a zygote happens during the transport through the Fallopian tube. On the fourth day after the ovulation the blastocyste arises out of the morula (32 cell stadium) by the inclusion of liquor. Thereby a cavity is formed with two compartments of cells around, the embryoblast and the trophoblast. The embryoblast is located in the centre and evolves into the embryonal body. On the other hand the outer cell layer, the trophoblast evolves into the placenta and the chorion.
The trophoblast now develops into two tissues, the inner cell layer, which is called the cytotrophoblast and is dividing activly, and the outer cell layer, the synzythiothrophoblast. During the implantation the first tissue penetrating the uterine epithelium is a multinuclear syncytiotrophoblast. The syncytiotrophoblast is a syncytial epithelium that functions as an endothelium when bathed in maternal blood.(Benirschke et al., 2012) The cells arising from cytotrophoblast proliferation differentiate and fuse with the synzytiotrophoblast. Thereafter the zona pellucida dissolves slowly and on the 7th to12th day after conception (p.c.) the implantation occurs as the embryonal pole of the trophoblast attaches to the compacta and spongiosa of the endometrium. The transformed endometrium is called the decidua and can be divided into 3 layers: the decidua basalis, capsularis and parietalis.
At the 8th day a small gap between the embryoblast and the trophoblast is developing, which is lined by a single layer of squamous epithelium and is called the amnion. Amnion and trophoblast are connected and this connection later becomes the umbilical cord. Simultaneously the embryoblast converts into a round germinal disk, which consists of two layers, the ektoderm and the entoderm.
Also from the 8th day on cells built of cytotrophoblast, line the inner layer of the blastocyste-cavity thus evoluting the primary yolk sack.

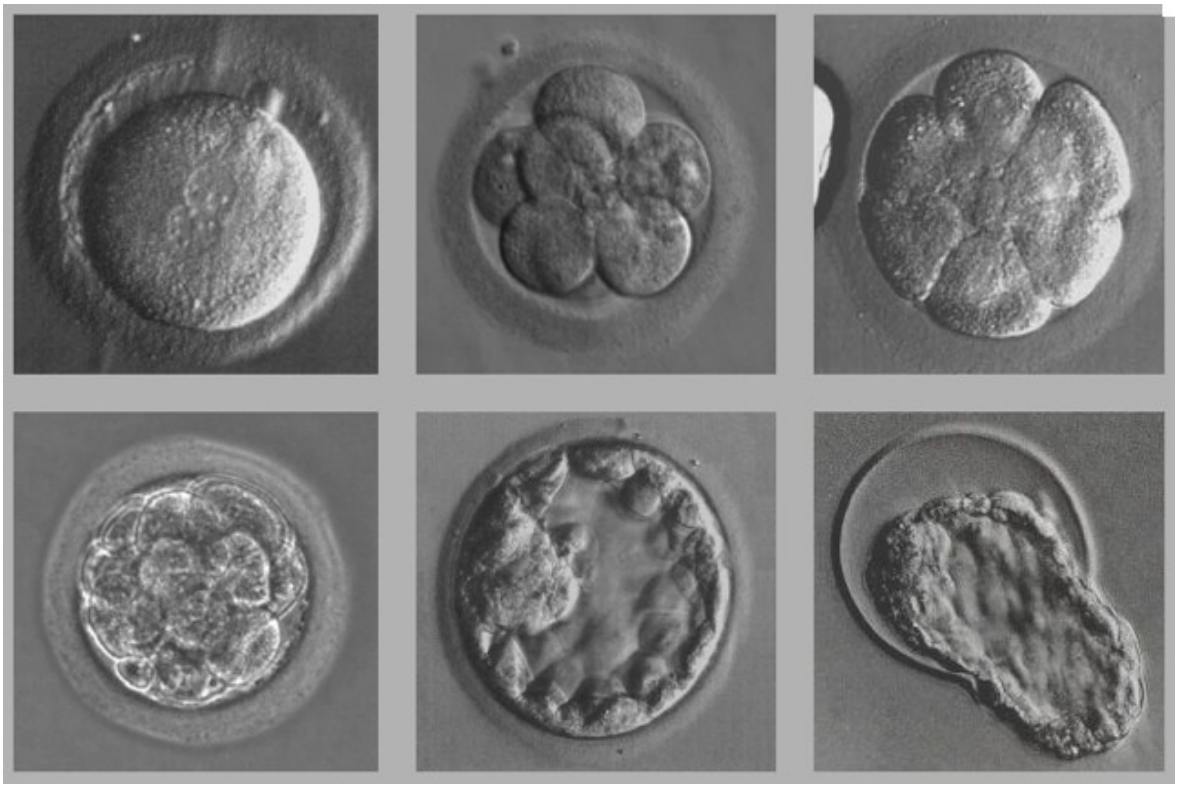

Figure 1 shows early human embryos:

top row:	left: fertilized egg	middle: 8-cell embryo (day 3)	right: cell adhesion
bottom row:	left: morula	middle: blastocyst	right: hatching embryo

Initially the supply of nourishment is ensued by the endometrial glands, which is called histiotroph nourishment, but from the 9th day on there arise lacunes in the synzytiotrophoblast which get filled out with blood from opened maternal capillaries. The maternal spiral arteries are transformed through the influence of invading trophoblasts and loose their smooth muscle coats. They also dilate to accomodate a tenfold increase in blood flow.

The syncytiotrophoblast is spongy bulked by building of lakunes on the 9th day. The trophoblast penetrates the maternal sinusoides and then maternal blood runs through the lakunes of the trophoblast.

So the fetus is connected to the maternal circulation and is nourished haematotrophic.

The implantation is completed on the 10th day. The embryo is implantated and now covered by the decidua and the gap of the uterine epithelium is closed by a blood clotting. On the 12th day the gap is closed and completely covered by the uterine epithelium.

After all, on the 13th day, the embryo consists out of three germinal sheets (ektoderm, entoderm and mesoderm) and the chorion cavity is built.

The amnion cavity grows and the yolk bag, which is lined with cells from the entoderm progressively minimizes. Now you can also recognize a third cavity, the chorion cavity, which is build out of trophoblast cells and mesoderm.
During the further development the amnion grows and puts over the embryo, so after all the amnion and the chorion are in thight contact.
From the 13th day on the primary villi develop by proliferaton of the cytotrophoblast. By growing of the mesenchym they change into secondary villi. And then there evolute capillaries in the centre of the mesenchym and so they become tertiary villi. Between chorion and decidua is a space filled with maternal blood, into which the villi extend from the chorion cover plate.
Around the 21th day placental vasculogenesis starts in the cores of secondary villi at the four somite embryo stage. For the evolution of the vascular system in the placenta vasculogenesis evolves into angiogenesis- these are sequentially occuring processes. Angiogenesis is the development of new vessels from already existing vessels and occurs from day 32 p.c. until delivery.("Aspects of human fetoplacental vasculogenes... [Placenta. 2004 Feb-Mar] - PubMed - NCBI," 2012) There are multiple aspects of vessel growth in angiogenesis, like branching and non branching angiogenesis. Non branching angiogenesis happens through elongation or intercalation, whereas branching angiogenesis is caused by sprouting or intussusception. So changes in the fetoplacental blood flow impact on the ratio of branching and nonbranching angiogenesis within the placenta. (Kay et al., 2011) Both processes are regulated by growth factors, paticularly VEGF, FGF and PIGF. The perfusion of the intervillous space starts in the peripher regions of the placenta.
Primary the chorion is beset with villi equally, but after the 8th week the villi degenerate on the side of the decidua capsularis, which is called chorion leave. Whereas the villi at the side of the decidua basalis proliferate and incorporate into the decidua. These villi become the chorion frondosum. This is caused by the relatively high oxygen concentrations at the abembryonal pole, where the amnion develops afterwards.The actual placenta develops from the parts which are not perfused until the first trimenon.
So the fetal part of the placenta develops out of the chorion frondosum and the maternal part is build of the deciduia basalis.
The fetal part of the placenta is limited by the chorionic plate, whereas the maternal side is covered by the decidua basalis which is adhered to the placenta and is called the basal plate.

There exist trophoblast cells as well as decidual cells in the area where the maternal and fetal parts are connected and so this is the place in which the cells of the trophoblast invade the uterine endometrium. There are located many so-called giant cells which are multinucleated trophoblasts found in the decidua, embedded in abundant glucosaminoglucanes. The intervillous space which is filled with maternal blood is situated between the chorionic and basal plate and arises from the former lacunes within the syncytiotrophoblast. According to that, the intervillous space is entirely lined with a syncytium, i.e. the syncytiotrophoblast. The placenta is dividetd into 10-38 lobes, the cotyledons, by decidual septae and every cotyledon consists of villous trees. The center of the septae consists of maternal tissue and their surface is covered by a layer of syncytiotrophoblast. The connection between adjacent cotyledons is maintained, because the septae do not reach the chorionic plate.

The amnion is covered by a tenuous part of the chorion- so it is called the amniochorionic membran.

At the 14th week of gestation the placenta has reached its final structure. The growth in girth is terminated with the 5th month, from there on there is only growth in surface. The growth is proportional to the growth of the uterus and is about 20-30 percent of the uterine surface. (Sadler, 2008)

The increase in thickness is caused by ramification of the already existing villi.

The embryonal blood system between mother and fetus is in place after 4-5 weeks p.c.. (Stauber and Weyerstahl, 2007)

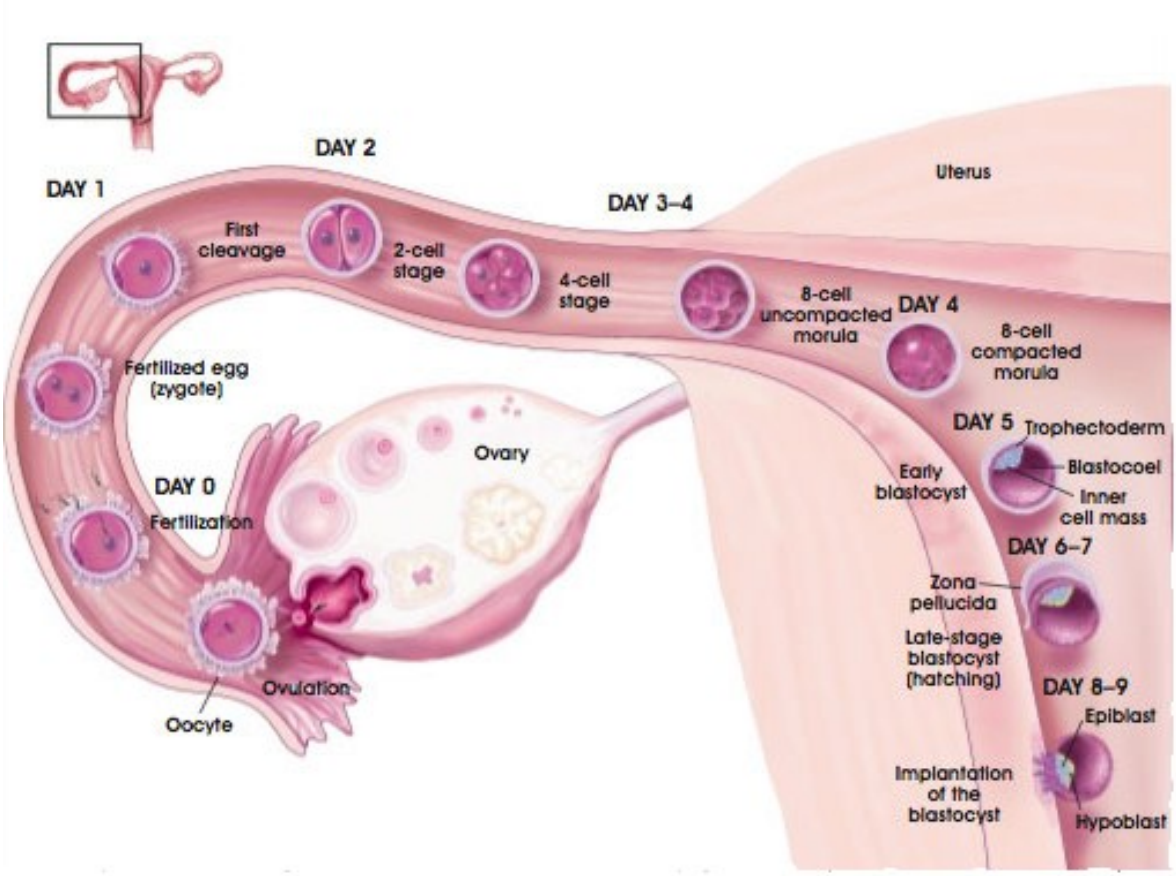

Figure 2 gives an overview of the stages in the journey from fertilization of the egg to implantation.

3.2 Development of the trophoblast

At the beginning of the second gestational month the placenta possesses many secondary and tertiary villi, which are arranged actinomorphic. The villi are anchored in the mesoderm of the chorionic plate and are connected peripherally to the maternal decidua by the outer cytotrophoblast. The surface of the villi consists of a syncytium, the so-called syncytiotrophoblast, which is overlying a layer of cytotrophoblast cells. This layer borders a mesodermal centre which is rich in fetal vessels. The capillary system of the villi associates with the vessels in the chorionic plate, which are connected to the fetal circulation by the umbilical arteries and veins.

The maternal blood reaches the placenta by the spiral arteries of the uterus. Cytotrophobasts erode the maternal vessels and so maternal blood is able to enter intervillous cavities. At the basal plate cytotrophoblasts invade the open confluences of the spiral arteries and so replace the maternal endothelium in the wall of the vessels so that these vessels are composed of fetal and maternal parts. Thereby the epithelial cytotrophoblasts fulfill endothelial functions .

The invasion of the spiral arteries by cytotrophoblasts remodel the spiral arteries as they become bigger, resulting in a decreased vascular resistance. Thus the mass of blood which streams to the intervillous spaces is increased.

Thereafter new villi branch out into the surrounding intervillous spaces. Primary the new built villi got the same layers as the stem villi, but at the beginning of the fourth gestational month the cytotrophoblasts and a part of the cells from the connective tissue recede.

From now on only the syncytiotrophoblast and the endothelium of fetal vessels seperate the maternal from the fetal circulation. The cytotrophoblasts first disappear in the small and then in the bigger villi, but a few are maintained in the stem villi.

The blood vessels which are located in the stem villi are not directly involved in materno-fetal gas and nutrion exchange. The syncytiotrophoblast gets very tenuous at many parts. The areas which carry nuclei bulge out and build up nodes. These nodes out of apoptotic multinuclear syncytiotrophoblast, so-called syncytial knots, can dissolve and so on they can be swept out in the maternal circulation. There they can stay in the pulmonary capillary system of the mother and mostly they dissolve without causing any symptoms. (Sadler, 2008)

So there can be distinguished different phenotypes of the trophoblast which perform different functions in the human placenta. Invasive extravillous trophoblasts detach from placental villi and penetrate the decidua basalis, where they transform spiral arteries to become so-called endovascular trophoblasts. Trophoblasts of the chorion leave modulate prostaglandin activity, and the villous trophoblast mediates maternal-fetal exchange and endocrine functions.(Kay et al., 2011)

3.3 Trophoblast Invasion

The invasion of maternal tissues and the anchorage of the placenta are the main functions of the extravillous trophoblast. Invasion is the process, in which the „extravillous trophoblast cells migrate from the basement membrane of anchoring villi and invade deeply, reaching the myometrium.“(“Matrix metalloproteinases and their inhibitors... [Gynecol Oncol. 2011] - PubMed - NCBI,” 2012)

In these processes the secretion of proteases, activators and inhibators is required.

There exist different populations of the extravillous trophoblast, which can be determined as outside the villous trees and are responsible for the uteroplacental connection.

Extravillous trophoblasts represent epithelial cells, which proliferate strictly out of cytotrophoblast progenistor cells on the basal lamina. After leaving the basal lamina, these cells leave the cell cycle and differentiate into an interstitial, apolar phenotype.

Those cells and their daughter cells, which do not proliferate afterwards, leave the basal lamina as they are pushed forward by the following generations of cells.

Later they begin to migrate actively in a matrix, which they secreted themselfes and become an invasive phenotype as last step of differentiation.

The trophoblasts which stay at the basal lamina can be seen as the progenistor cells of the extravillous invasive pathway and are proliferative (so called Langerhans cells), whereas the invasive trophoblast cells are not.

Cells in close proximity to the basal lamina are less differentiated than those in increasing distance where they get more and more differentiated.

During the differentiation the extravillous trophoblasts undergo different phenotypes such as proliferative progenitors, early postproliferative cells and small spindle-shaped cells. Moreover, they can also differentiate into multinucleated giant cells or large polygonal (X-cells).

Thus, there are noninvasive large polygonal cells as well as highly invasive spindle-shaped cells during the invasion.

The small spindle-shaped highly invasive trophoblasts can be shown by the expression of integrins and their structue, and by the distribution of these cells, which is homogenously from the first third of the myometrium to the distal parts of the cell columns. Even their shape reflects and enhances movement and invasiveness-comparable to invasive tumor cells.

The large polygonal extravillous trophoblast cells are assumed to be derived by those spindle-shaped cells and are less invasive or even noninvasive. They ususally are more frequent in the superficial layers of the basal plate and they increase in number as much as the gestation advances. This suggests that invasiveness is reduced in that way.
It is assumed, that the invasive pathway could be associated with the variation of the oxygen partial pressure at the fetomaternal interface during gestationa. In early pregnancy there is a very low oxygen level in the intervillous space, but at about week 12 the intervillous perfusion with maternal blood increases and thereby the oxygen pressure rises. There are different studies, addressing the question whether hypoxia stimulates or reduces the invasiveness of the trophoblast cells. The conclusion is not distinct, but it was demonstrated that the oxygen partial pressure plays an important role in regulation of the invasiveness and it is assumed, that this role is more likely a stimulating one.
However, the invasion of trophoblasts is not only regulated by oxygen. There are also mechanisms induced by the desidua to "defend" itself, i.e. invasion depth has to be controlled very strictly. Thus, penetration of the placental bed reaches to the inner third of the myometrium in normal pregnancies.
There exist different features that promote or limit trophoblast invasiveness, including decidual activities, growth factors, MMPs and immunologic mechanisms.
Especialy in the first trimenon of gestation a diabetic pregnancy has a higher risk for missed abortions. It is assumed, that this is caused by a failure of trophoblast invasion.(Kay et al., 2011)

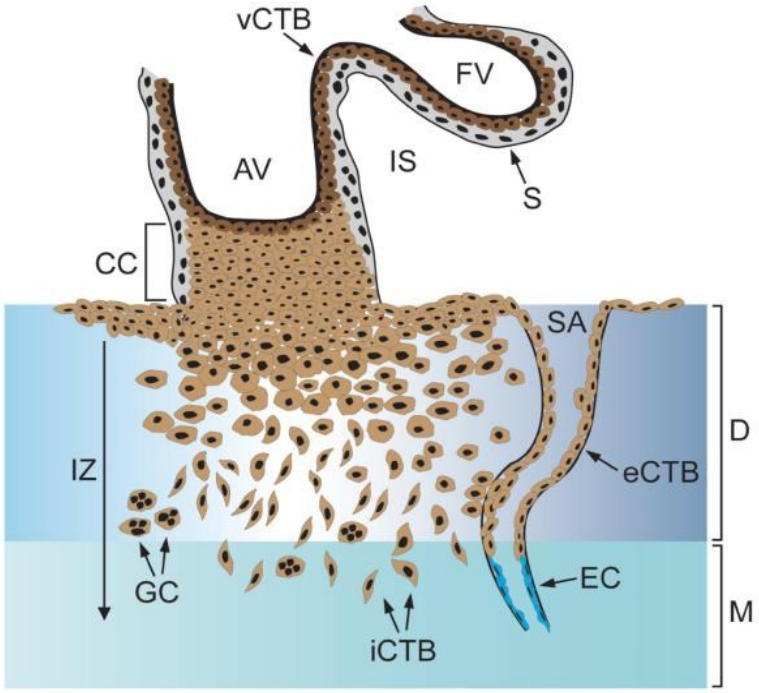

Figure 3 : Trophoblast invasion

This figure shows the invasive process of the trophoblast cells. First of all the mesenchymal villus penetrates the basal membrane and the cells proliferate in lines of columns (CC). The maternal layer of those cell columns develop into non-proliferative cells which are called extravillous because they migrate to the stroma of the maternal decidua (D). This means that they can now be called interstitial cytotrophoblasts (iCBT). The intersitial trophoblast cells differentiate into giant cells (GC) which are located in deeper areas of the decidua stroma. The endothelial cells (EC) of spiral arteries (SA) are replaced by the endovascular cytotrophoblast cells (eCTB) which then function as an endothelium.
The multinucleated syncytium (S) is built by the fusion of cytotrophoblast progenitor cells which are located in the floating villi (FV) and are surrounded by maternal blood. The maternal blood fills out the intervillous space (IS) and enable the exchange of oxygen and nutrients

3.4 Structure of the human placenta

The shape of the human placenta is discoidal and and the materno-fetal connection is characterised by villi. There exist different types of villi: mesenchymal, immature and mature intermediate, stem and terminal villi.

The basic villous structure consists of a surface layer of syncytiotrophoblast, a subjacent layer of villous cytotrophoblasts, a basement membrane that delimits a connective tissue villous core, which contains tissue macrophages called Hofbauer cells and an arcade of fetal vessels. (Kay et al., 2011) The types of villi can be distinguished by their different compositions:

The mesenchymal villi have a relatively dense stroma, small unimposing vessels and are rich of cells. The immature intermediate villi have many macrophages and small vessels, a loose stroma and ducts in the stroma, whereas the mature intermediate villi do not have ducts in the stroma and only little, marginal capillaries. Evaginations of the mature intermediate villi are the terminal villi. They develop from endothelium in the mature intermediate villi, which expands faster than the surrounding connective tissue. This happens during the second and third trimenon. The main function of terminal villi is exchange of gases, nutrients and metabolites between the maternal and fetal circulation. The stem villi are characterised by big vessels in the centre, a stroma with low cell density, but rich in fibres, vascular plane muscle cells and exravascular myofibroblasts. The stem villi provide mechanic stabilisation like the trunk of a tree.

Over gestation the core of the villi changes: primary the villi have little connective tissue, which faciliates the recognition of the Hofbauer cells. They are edematous with fluid channels and limited fetal vessels. There occur also undifferentiated stromal cells, myofibroblasts, fibroblasts and pericytes in the cellular composition of the stroma. In later gestation the villous core contains more cells, lesser amounts of collagen, more vessels and offers a diminished diffusion barrier for gases and nutrients to transfer between the maternal and fetal circulation. (Kay et al., 2011)

The villous trees which form a cotyledon are surrounded and limited by septae consisting of decidua which was invaginated. The septae are built by tissue of the basal plate. Every cotyledon is supplied with fetal blood by a chorionic vessel. The cotyledons are bathed in maternal blood, which arises from the spiral arteries and can exchange oxygen and nutrients with the fetal blood. At the chorionic surface the ramification of the umbilical vein and the two umbilical arteries takes place. Thereby the veins run under the arteries.

In most placentas the umbilical cord does not insert in the centre of the placenta at the chorionic plate, mostly its eccentrically. It consists of the ductus omphaloentericus and its vessels, the former adhesive stem with the diverticulum of the allantois and the Aa. and V. umbilicales.

The placental weight is on an average of 470g, but the weight is directly proportional to the weight of the fetus at the ratio of seven (fetus to placenta). (Kay et al., 2011)

The cross-section dimension of the placenta is about 15-25cm and the mean thickness in the center of the placenta is 3cm.(Sadler, 2008)

The amniotic cavity is encompassed by the chorioamnion membrane. This membran develops by the fusion of the chorionic and the basal plates at the margins of the placenta.

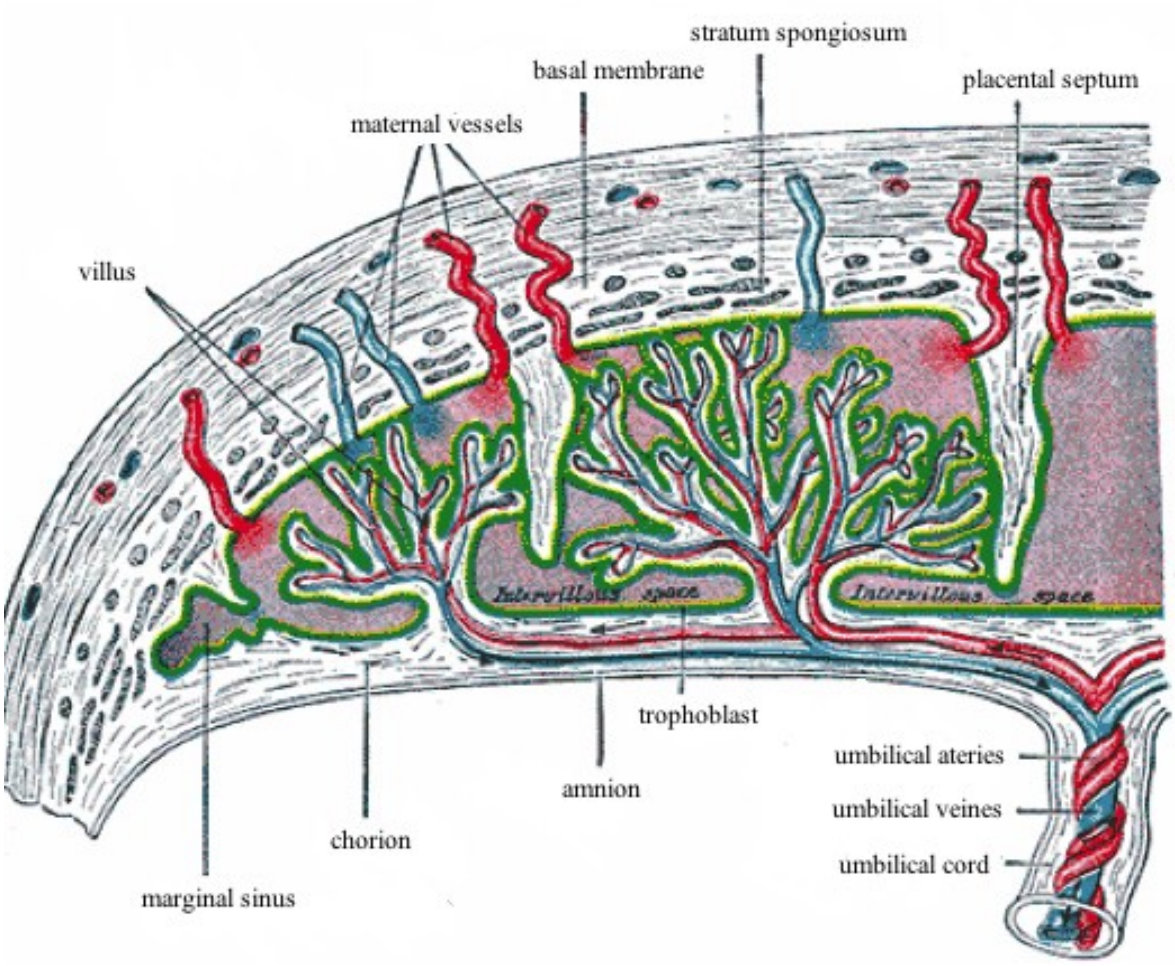

Figure 4: Structure of the human placenta

3.5 Function of the human placenta

Essentially the human placenta is the organ of exchange most notably for oxygen and carbon dioxide, the nutritients and metabolites. However, there is also exchange of drugs, blood cells (erythrocytes and leukocytes by diapedese) (Dudenhausen, 2011) and immunoglobins. This results from active transport or passive diffusion according to the substrates' physic-chemical nature. Aktive transports like pinocytosis is energy dependent. On the contrary passive transport follows a concentration gradient and is thus is energy independent. Moreover, cells and other compounds may pass by diapedese through channels in the syncytiotrophoblast and intercellular gaps between cytotrophoblasts and vascular endothelial cells.

Ther maternal blood leaves the spiral arteries of the decidua basalis and incorporates the intervillous cavities of the placenta. First of all it arrives at the chorionic plate under high pressure, wherefrom it relapses at the basal plate and bathes the cotyledons. This enables an intensive gas and mass transfer to the fetal blood. Finally it circulates to the maternal veins via passage through the utero-placental veins. The fetal blood, which is low in oxygen and charged with degradation products, reaches the placenta by the two umbilical arteries. The arteries branch at the chorion plate and lead to the cotyledons as so called strain arteries. The indirect contact with the maternal blood on the surface of the villi, which is overall 10-15 m^2. ("Regulation of vascular growth and function in the human placenta," 2012) , happens in the spacious capillary network. The interlayer between maternal and fetal circulation – the placental barrier – consists of fetal vascular endothelium and its basal lamina, the connective tisue in the centre of the villi, the cytotrophoblast and the synztiotrophoblast up to the fourth month. Afterwards the placental barrier gets more tenous, because the cytotrophoblasts abolish and the fetal capillaries attaches straight to the syncytial layer. This way the transport and diffusion distance is brought to a minimum, which facilitates the exchange.

The human placenta is called haemo-monochorial, because the barrier layer between maternal and fetal blood in the intervillous cavity consists of chorion where a single layer of trophoblast cells separates the maternal blood from the capillaries of the foetus.

The delivery of fetal metabolites like carbon dioxide, urea and bilirubin as well as the ingestion of oxygen and nourishing substrats happens through the placental barrier.

The nourishing substrates arrive to veins of the cotyledons and reach the fetus through theV. umbilicalis .

The intervillous space of the mature placenta contains about 150ml of blood, which exchanges three to four times per minute. This blood bathes the chorionic villi, whose surface is about 4-14 m^2. But only the villi whose fetal vessels stay in tight contact with the syncytial layer , particicipate in the placenal exchange.(Sadler, 2008)
The intervillous pO_2 of the placenta is extremely low (pO_2 <15 mmHg) (Kay et al., 2011) in the first trimenon compared to the second and third trimenon, because cells divide even faster at this condition. But a reduction in oxygen also reduces the number of reactive oxygen and as a result of that the risk of damages in the DNA.
The intervillous pO_2 in the first trimenon is about 2-3%, increases to 7-8% in the second trimenon and finally deacreases to 5-6% in the third trimenon. The values in the second trimester are similar to the pressures of the circulation in the veins or capillaries in the other parts of the body.(Kay et al., 2011)
The human placenta also produces hormones, which are proteohormones: human chorionic gonadotropin (hCG), human chorionic thyrotropin (hCT), human placental lactogen (hPL) and steroidal hormones (gestagenes and estrogenes). However, the synthesis of steroid hormones depends on steroid precursors which are synthesized by the fetus or the mother. (feto-materno-placental-system).
At the end of the fouth month the placenta is producing sufficient progesteron by its own to maintain the pregnancy, even if the corpus luteum recedes or is dysfunctional.
The production of estrogenes reaches its peak at the end of the pregnancy and stimulates the growth of the uterus and the mammary glands.
The fetus has the priority in relation to the blood sugar level. Thus a maternal, diabetic metabolism can be favoured. This is regulated by the human placental lactogen (hPL), which is nowadays called somatomammotropin. It also stimulates the growth of the mammary glands.
The exchange of the products of metabolism like amino acids, free faty acids, carbohydrats and vitamins as well as electrolytes increases with progression of pregnancy.
The immunological capacity develops at the end of the first trimester. At this moment the fetus is able to build all components of the complement system. The main fraction of fetal immunoglobins is maternal IgG, which passes to the fetus from the 14th week of gestation. Thus the fetus is immunized passively. The fetal production of immunoglobins starts postpartum and reaches the adult blood levels at the age of three.
In the pregnant uterus the innate immune system predominates, likely to secure tolerance to the fetus.(Kay et al., 2011)

The amniotic cavity is filled with clear, aqueous liquor which is produced by the cells of the amnion. This liquor increases in volume from about 30ml at the 10th week to 350ml at the 20th week and finally to 800-1000ml in the 37th week.(Sadler, 2008)
In the first month of the pregnancy the amniotic liquor serves as a protective barrier for the fetus. It also prevents adhesive processes between the fetus and the amnion. Furthermore the amniotic liquor faciliates fetal movement and helps at the process of the delivery.
The amniotic liqour is exchanged every three hours - this shows the high shifts of liquids between maternal circulation and the amniotic cavity.
The fetus drinks about 400ml of the amniotic liquor every day, this starts at the 5th month. Afterwards it is reabsorbed by the fetal intestins and brought back to the maternal circulation by the placental blood flow. At the end of the pregnancy the fetus excretes urine into the liquor-but only in low concentrations- the placenta still is the organ of excretion.(Sadler, 2008)

3.6 Significance and Prevalence of Diabetes mellitus

In the first world countries the development of diabetes due to the lack of physical movement and increase in consumption of fast food leads to an enhancement of Diabetes. The prevalence of Diabetes-that includes type I, type II and gestational diabetes mellitus-is about -3% to 20% of pregnant women. (Kay et al., 2011)

Before insulin has been discovered nearly no pregnancies in women who suffered from Diabetes were possible, because those women were infertile and amenorrheal. If, however, pregnancy occurred, the mortality and risk of complications were very high.

Diabetic conditions lead to problems in pregnancy as fetal growth restriction and macrosomia are frequently observed pregnancy complications. There is also an enhanced risk of abortion, preeclampsia and eclampsia, acute respiratory distress syndrome, gestosis and hydramnion. ("Pregnancy in Type 1 Diabetes Mellitus: How Special are Special Issues?," 2012)

Gestational diabetes mellitus (GDM) is associated with an increase in perinatal mortality and morbidity. Long-term consequences for the offspring born to a GDM pregnancy have been predicted resulting in endothelial dysfunction associated with hypertension, obesity, type 2 diabetes mellitus (T2D), and metabolic syndrome. ("The placenta and gestational diabetes mellitus. [Curr Diab Rep. 2012] - PubMed - NCBI," 2012)

So this leads to the question how the human placenta as the organ between the maternal and fetal circulation changes under diabetic conditions.

3.7 The human placenta in diabetes

In diabetic conditions the maternal and the fetal circulation changes, because hormones, nutrients and growth factors will be influenced.
There is a morphologic and functional alteration of the placental development and as a consequence also of the fetal development.
Primary there is an elevated blood sugar level in the maternal circulation which will be transported to the fetal circulation. This causes a hyperstimulation of the fetal pancreas and as a consequence of that the fetus will produce more insulin which in turn leads to hyperinsulinemia. Thereby the aerobic metabolism is stimulated which results in an increased oxygen demand. This may lead to placental hypervascularization (increased calibre and number of blood vessels) and fetal hypoxia. The fetal hypoxia is aggravated by a reduced oxygen transport to the fetus, because of placental changes like thickening of the basement membrane. Chronic hypoxia or hypoxemia causes an increased number of terminal villi and capillay loops, because the endothelial proliferation is altered. Thereby the villous surface area is increased to enhace the fetal oxygen and nutrient supply. The placental vasculature does not posses precapillary sphincters or regulatory arterioles to preserve perfusion pressure. So there is no possibility of dilution in case of low oxygen levels. Additionally, term placenta tissue is not able to form new capillaries which are positioned in parallel with the already existing ones, but there rather happens the development of capillary loops through intercalation. The problem thereby is that vascular resistance can not be decreased by capillaries which are not in parallel with the already existing ones. So those capillary loops have a negative influence on the fetoplacental blood flow and are not able to compensate hypoxic conditions.

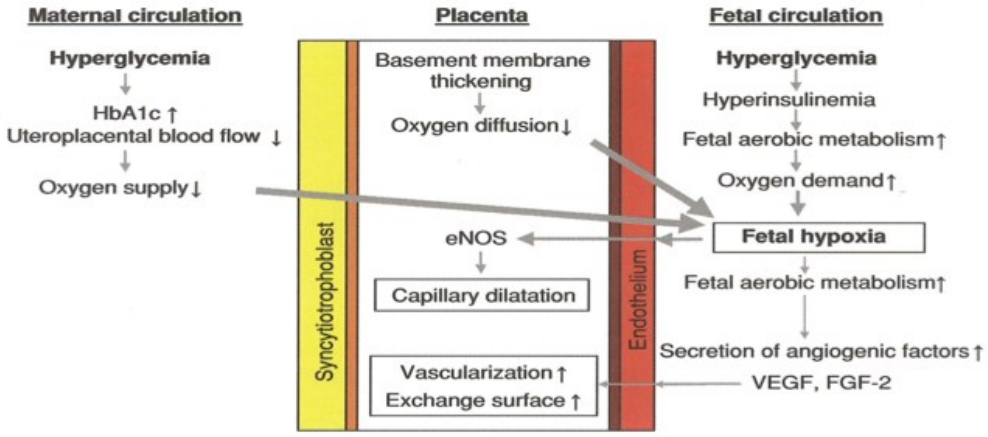

Figure 5 gives a summary of the changes in maternal and festal circulation as well as in the placenta in diabetic conditions.

The best clinical indicators of alterations that could cause hypoxia are the Doppler indices of blood flow and resistance to blood flow.(Kay et al., 2011)

"With regard to abnormal placental development, it is hypothesized that when cytotrophoblasts do not access an adequate maternal blood supply, the cells are less able to invade the placental bed, due to the hypoxic environment. This decreased invasion sets up events resulting from shallow placentation, such as IUGR, fetal death, or preeclampsia."("Hypoxia inhibits invasion of extravillous trophobla... [Placenta. 2011] - PubMed - NCBI," 2012)

However, recent data suggested no association between fetal growth restriction or preeclamsia and hypoxia in the placenta. Placental injuries like defects of the trophoblast invasion into the spiral arteries may happen by affecting the diameter of the capillaries. They may get smaller in diameter and the maternal blood may have to enter at higher velocity. This may harm the fragile villous trees.("Rheological and physiological consequences of conve... [Placenta. 2009] - PubMed - NCBI," 2012)("Placental origins of preeclampsia: challenging ... [Hypertension. 2008] - PubMed - NCBI," 2012)

Also the expression of transferrin receptors will get increased and this causes a stimulation of the haemoglobin sythesis to compensate the fetal hypoxia. ("Histopathological placental lesions in mild gestational hyperglycemic and diabetic women," 2012)

Those changes of placental function and development are also caused by altered levels of leptin, cytokines and TNFα and may even occur before clinical symptoms manifest. ("Abstract - SpringerLink," 2012)

TNFα for example is a cytokine, which is antiangiogenic and inhibits the invasion of the trophoblast. This effect may be counterbalanced by leptin, which stimulates the invasive processes and angiogenesis. (Kay et al., 2011)

Hyperinsulinemia also influences growth factors, which induce the adipocytes to increase fat accumulation (lipo- and mitogenic) (Kay et al., 2011) and this leads to fetal macrosomia. The definition of macrosomia is a birth weight above 4000 grams or a better characterization: a growth which is disproportionate for size ("Large for gestational age - Wikipedia, the free encyclopedia," 2012) and this brings along problems and complications not only in the pregnancy but also for the delivery.

In the first Trimester of the pregnancy the trophoblast proliferation, differentiation and invasion may be altered and that could cause alteration in placental anchoring and therefore missed abortions. Especially in the first trimester of gestation it is assumed that there is a higher risk of abortions caused by pregestational diabetes. Aditionally levels of MMPs are altered, which can be seen as markers for tissue remodeling during the invasion of the trophoblast. ("Insulin and the IGF system in the human placenta of n... [J Anat. 2009] - PubMed - NCBI," 2012)

The consequence of altered invasion in early pregnancy is a reduced uteroplacental blood flow and this may cause predispositions for intrauterine growth restriction and/or preeclampsia. Notably, recent studies demonstrated a direct correlation between increased maternal HbA1c levels in the first trimester and the development of preeclampsia.

Under diabetic conditions the trophoblast basement membrane will get thicker and the number of cytotrophoblasts will increase. (Kay et al., 2011) However, those alterations always depend on the time of onset, the duration of the changed environment caused by hyperglycemia and also on the maternal body weight, metabolic status, severity of diabetes and therapy.

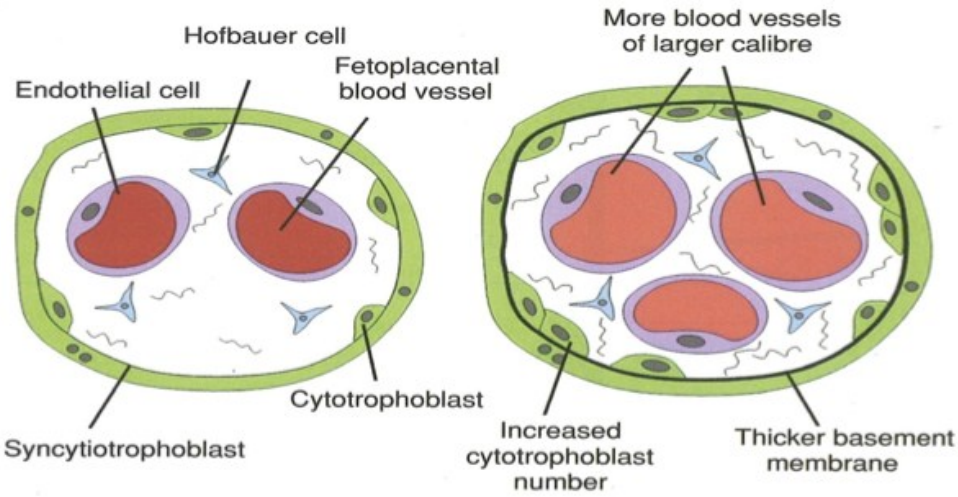

Figure 6 shows a schematic cross sections through placental villi at term of gestation from normal (left) and diabetic (right) pregnancies. In diabetes the surface area is increased, the trophoblast basement membrane gets thicker and the number of blood vessels and cytotrophoblasts increases. Also the calibre of the blood vessels is increased as well as the number of extracellular matrix proteins. The fetal hypoxia in diabetic pregnancies is indicated by the light red colouring of the vessel lumen.

Oxygen is a major modulator of placental endothelial growth, and a low oxygen environment is crucial for the onset of vasculogengesis early in placental development. Although therapies are advancing in the management of diabetic women, the problems and histopathological changes like macrosomia, increased surface area, edema and hypervascularization of the placenta still could not be normalized yet. (Kay et al., 2011)
It is assumed, that the diabetic placenta, whose stores buffer capacity normally is lower than that for fetal skeletal muscle, can increase its glycogen depots due to elevated maternal levels of glycogen, phospholipids and triglycerids. These stores have been found primarily around the fetoplacental vessels.
The lipid accumulation in the diabetic placenta may contribute to the fact that diabetic fetuses mostly are macrosomic. This may be explained by a higher availability in nutrients for the fetus.

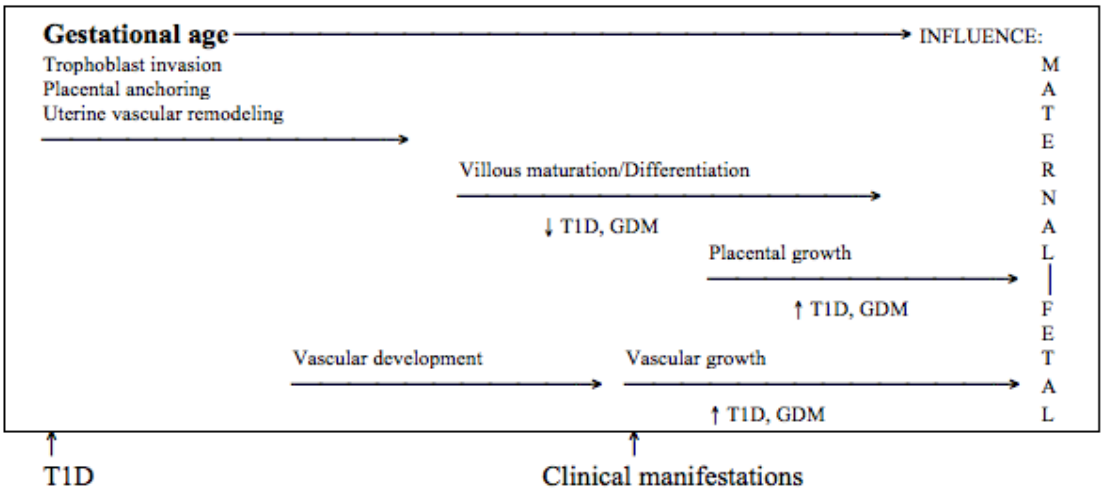

Figure 7 shows the dependence of the moment of the diabetic insult to the effects on the organism. For example in early pregnancy the risk of preeclampsia, growth restriction or pregnancy loss can be increased because of failures in trophoblast invasion.
Maternal alterations in the blood may influence the trophoblast cells, because they are in direct contact with it, whereas fetal alterations in the blood may influence the vasculature of the placenta.
Type I diabetes mellitus (T1D) as well as GDM may have an effect on the development of the placenta, but it also depends on the moment of the insult and the manifestation of diabetes.

Another aspect is that trophoblast invasion has an impact on the uteroplacental blood flow as maternal spiral arteries get opened and remodeled into low-resistance vessels. Another influencing factor are villus edema, hypervascularizated and more bulbous villi which leads to a reduction of the intervillous space. Inadequate opening of spiral arteries and a reduction of estradiol, which is a vasodilator synthesized by the trophoblast has also been observed in diabetic placentas. Changes in these processes may lead to decreasing maternal blood flow into the intervillous space. This could as a consequence also lead to a compromised fetal blood flow.
In all those mentioned facts the type of diabetes as well as the gestational period during which the diabetic alterations occur, may play a significant role. (Kay et al., 2011)(“The Human Placenta in Gestational Diabetes Mellitus,” 2012)

3.8 Oxygen as a modulator of trophoblast invasion

In the first trimester, one key regulator of the number of extravillous trophoblast cells is oxygen. This low oxygen environment, prior to the onset of maternal blood flow into the intervillous space, is suggested to be obligatory for the development of embryo and placenta as there is an association of low oxigen environment to reduced levels of reactive oxygen species and thereby less DNA damage as well as lower invasiveness and higher proliferative activity of the trophoblasts. This high rate of proliferation is needed to achieve the rapid growth of the placenta at that stage of development.

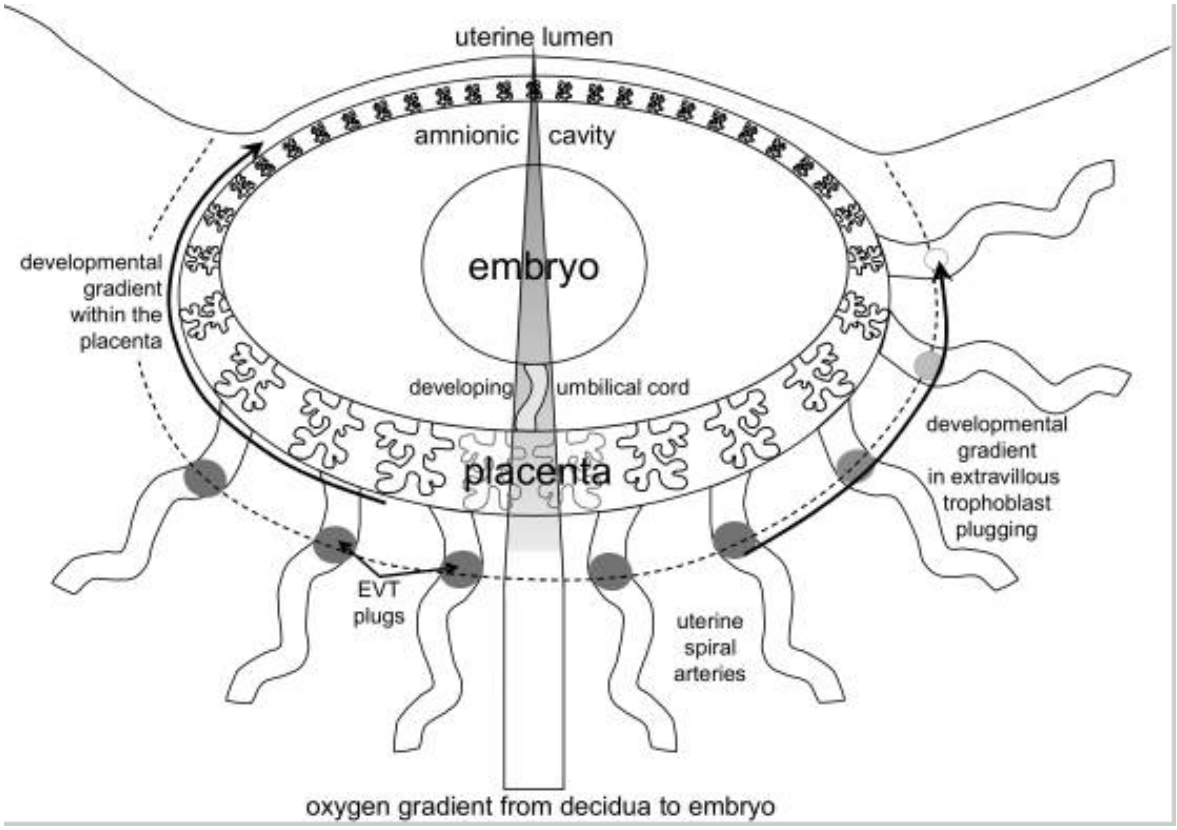

Figure 8: Schematic representation of an embryo and a placenta at about 8-10 weeks of gestation

There exists an oxygen gradient from the decidua towards the developing embryo. During the first trimester the pO_2 in the placental bed is about 70 mm Hg, but the gradient in the oxygen concentration from the decidua to the placenta, results in a pO_2 in the placenta of less than 20 mm Hg and the embryo still less than 20 mm Hg. This oxygen gradient is represented by the bar in the centre. The highest level of placental development is at the site of implantation (arrow on the left) at the embryonic pole. The arrow on the right shows the developmental gradient in extravillous plugging. The dotted line indicates the plugging of spiral arteries which generates the oxygen gradient towards the embryo.

With the onset of maternal blood flow, the oxygen gradient from decidua to embryo decreases. This change in oxygen leads to alterations in the rates of apoptosis and/or proliferation of extravillous trophoblast and thereby generation of a large number of invasive cells.

An increase in oxygen before the normal onset of maternal blood flow into the intervillous space may cause a loss in placental mass or even spontaneous abortion, because this may damage the stem cell pool of extravillous trophoblast cells in the proximal parts of the cell columns which are highly proliferative and rest on the basement membranes of anchoring villi. These cells show high rates of proliferation during the first trimester under low oxygen, but reduce their generative potency and number of cells available to invade maternal tissues in an early onset of maternal blood flow leading to a premature rise in oxygen. ("Oxygen as modulator of trophoblast invasion," 2012)

Any changes in the amount of invading trophoblasts may affect regular remodeling of uteroplacental arteries and thereby may restrain maternal blood supply to the placenta and again have pathological effects on the growth of the fetus. ("Oxygen as modulator of trophoblast invasion," 2012)

Oxygen tension rises evermore during the first trimester of gestation and leads to increased production of reactive oxygen species (ROS). This may be caused by oxygen which modifies hyperglycemic effects on ROS formation. The result is a decreased first-trimester trophoblast growth. A study with a first trimester trophoblast-derived cell line (ACH-3P) cultivated in normoglycemia at 2.5%, 8%, and 21% oxygen or in hyperglycemic conditions for up to 3 days could show a reduced cell number by 65% (under hyperglycemic conditions) and resulted in changes of the cell cycle (G(1)- and S-phase) at 21% oxygen. Moreover, the study showed that intracellular ROS was elevated under hyperglycemic conditions independent of the oxygen concentration, whereas mitochondrial superoxide levels were enhanced under hyperglycemia at 21% oxygen. The combination of hyperglycemia and high oxygen levels (21%) reduced the proliferation of human trophoblasts in first-trimester placentae. Reduced placental growth and accordingly embryonic growth during the first-trimester, as observed in pregestational diabetic pregnancies, may be associated with an increased oxygen tension in first trimester pregnacy. ("Oxygen modulates the response of first-trimester... [Am J Pathol. 2012] - PubMed - NCBI," 2012)

3.9 The Matrix metalloproteinases (MMPs)

MMPs are a group of enzymes, which catalyse the proteolysis of peptide bondings. For this reason they are called peptidases. More precisely they are a group of zink-dependent endopeptidases, which are structurally related to each other.("LEF-1 regulates proliferation and MMP-7 transcription in breast cancer cells - Bucan - 2012 - Genes to Cells - Wiley Online Library," 2012) They regulate a broad panel of biological functions("Specificity of binding with matrix metalloproteinases. [EXS. 2012] - PubMed - NCBI," 2012) and are located in the extracellular matrix, where they can remodel tissues after having been activated. This happens during angiogenesis, growth of tumors, wound repair and also the invasion of trophoblasts. These proteolytic enzymes are essential for the invasion, because they enable the migration of the extravillous trophoblast cells into the endometrial stroma. They also play a role in the remodeling of connective tissues which occurs in normal and pathological processes. The family of human MMPs consists of 14 members which can be classified into four different families: stromelysins, membrane-type MMPs (MT-MMPs), collagenases and gelatinases. Apart from those there are some enzymes like stromelysin-3 and macrophage metalloelastase that do not belong to these groupings. ("Identification and characterization of a novel h... [J Biol Chem. 1997] - PubMed - NCBI," 2012)
MMPs are able to cleave the proteins in the extracellular matrix with the result that the extracellular matrix is given an opportunity to renew and to transform. ("Matrix metalloproteinases and their inhibitors... [Gynecol Oncol. 2011] - PubMed - NCBI," 2012) They are secreted by trophoblasts, showing immunoreactivity with increasing depth in the surrounding extracellular matrix and also intercellularly.
In addition, MMPs are also able to break down proteins beyond the extracellular matrix. Thereby they can play a role in tumor cell invasion and metastasis as well as other pathologies like inflammations.("Matrix metalloproteinases and their inhibitors... [Gynecol Oncol. 2011] - PubMed - NCBI," 2012)

3.9.1 MMP 2

The mRNA of this proteinase was found only in the invasive phenotype (Polette et al., 1994), i.e. in extravillous trophoblasts , where its expression has been demonstrated by immunhistochemie , in situ hybridization (Autio-Hermainen et al., 1992; Fernandez et al., 1992; Blankenship & King, 1994b; Polette at al., 1994; Huppertz et al., 1990) and also in *in vitro* approaches (Emonard et al., 1990).

MMP 2 degrades mainly collagen IV, which is the major structural component of basement membranes. It is a gelatinase and has also been regarded as „the key enzyme in placentation"("Hypoxia inhibits invasion of extravillous trophobla... [Placenta. 2011] - PubMed - NCBI," 2012).

MMP 2 is suggested to play an important role in the invasion of trophoblasts in early stages of pregnancy and it is located in the placental bed continuously in early pregnancy. Studies demonstrated the correlation between decreased MMP 2 expression and inadequate invasion of extravillous trophoblasts caused by hypoxia in the first trimester of gestation. ("Hypoxia inhibits invasion of extravillous trophobla... [Placenta. 2011] - PubMed - NCBI," 2012)

3.9.2 MMP 7

This Matrix metalloproteinase, which is the smallest of all MMPs ("LEF-1 regulates proliferation and MMP-7 transcription in breast cancer cells - Bucan - 2012 - Genes to Cells - Wiley Online Library," 2012), is also found in the decidua and the invasive phenotype of the trophoblast . There has been shown an overexpression by the invasive trophoblast cells in preeclmapsia.(Vattraino et al., 1996)

It is produced by epithelial cells and is able to brake down elastin, proteoglycans and fibronectins.(Benirschke et al., 2012)

MMP 7 is called matrilysin and is able to generate angiostatin by hydrolyzing plasminogen (as well as MMP-9). This way it is involved in the regulation of angiogenesis, because angiostatin can inhibit tube formation, endothelial cell migration, proliferation and can induce apoptosis. But also through the induction of angiogenic inhibitors like arrestin, tumstatin, canstatin or endostatin, which are localized in the extracellular matrix. ("Low microvascular density at the tumor cent... [Int J Clin Oncol. 2012] - PubMed - NCBI," 2012)

It is expressed in the occurence of tumor invasion and metastasis of different types of cancers including breast, ovary, lung and prostate cancer. Several studies showed that in normal aging of the human breast the expression of MMP 7 decreases while compounds of the extracellular matrix increase. In contrast to that MMP 7 was found upregulated in invasive breast cancer cells and it is also called a „metastasis-associated gene"("Down-regulation of matrix metalloprotein... [Clin Exp Metastasis. 2012] - PubMed - NCBI," 2012).

Literate data also indicate that MMP 7 may represent a target gene in colorectal cancers. ("LEF-1 regulates proliferation and MMP-7 transcription in breast cancer cells - Bucan - 2012 - Genes to Cells - Wiley Online Library," 2012)

3.9.3 MMP 9

MMP 9 is a collagenase/gelatinase B, which mainly degrades collagen IV. It was demonstrated on mRNA and protein levels in the proliferating layers of the extravillous trophoblast cells (Polette et al., 1994). In the early invasive stages the expression is downregulated and afterwards upregulated in the deeper invasion stages (Huppertz et al., 1998d). There has been shown that an antibody against MMP 9 could completely inhibit the invasion of the trophoblast (Librach et al., 1991b; Polette et al., 1994). In culture supernatants of trophoblast cells from preeclampsic placentas showed a reduced MMP-9 activity. (Graham et al., 1996) (Benirschke et al., 2012)

Another study showed an enhanced expression of the MMPs 7 and 9 in liver tumors ("Silibinin Inhibits Tumor Growth in a Murine Orthotopic Hepatocarcinoma Model and Activates the TRAIL Apoptotic Signaling Pathway," 2012) and MMP-9 is also mentioned as an „osteoclast-related" gene. ("Quercetin Triggers Apoptosis of Lipopol... [Cell Physiol Biochem. 2012] - PubMed - NCBI," 2012)

It is also suggested that the expression of MMP 9 is a mediator in tumor invasion and metastasis where it is found to be overexpressed. ("The transcription factor SPDEF suppresses prosta... [J Biol Chem. 2012] - PubMed - NCBI," 2012). Moreover it may be involved in the remodeling of the porcine neonatal cervix by extracellular proteolysis. ("Nursing During the First Two Days of Life Is E... [Endocrinology. 2012] - PubMed - NCBI," 2012)

Another study, which compared placental tissue from healthy with gestational and pre-existing diabetes mellitus pregnancies could show a higher MMP 9 activity in placentas from the patients with pre-existing diabetes mellitus. In contrast, placental tissue from the group of patients with gestational diabetes mellitus showed a decreased MMP-9 activity. The reason may be found in the regulatory effect of NO and ROS on MMP 9, so that functional and structural abnormalities of diabetic placental tissue may be caused by changes in the MMP 9 activity modulated by ROS and NO.("Membrane-type matrix metalloproteinase-9 a... [Reprod Fertil Dev. 2000] - PubMed - NCBI," 2012)

3.9.4 MMP 19

MMP 19 is suggested to degrade several basement membrane proteins like laminin 5 γ2 chain, tenascin C, type IV collagen and nidogen-1. This capacity and its expression pattern may indicate the role of MMP 19 in angiogenesis and vascular remodeling. ("BMC Biochemistry | Full text | Matrix metalloproteinase-19 inhibits growth of endothelial cells by generating angiostatin-like fragments from plasminogen," 2012)

It is expressed in various tissues including ovaries, intestine, pancreas, lung, spleen and the placenta and may have a special purpose there. ("Identification and Characterization of a Novel Human Matrix Metalloproteinase with Unique Structural Characteristics, Chromosomal Location, and Tissue Distribution," 2012)

MMP 19 is a protein which shows the domain structure characteristic of all other members of the MMP family. This includes a prodomain with the cysteine residue which is a signal sequence essential for maintaining the latency of these enzymes, a COOH-terminal fragment with sequence similarity to hemopexin and an activation locus with a zinc-binding site. But it is deficient in structural features of diverse MMP subclasses, like the Asp, Gly and Tyr residues. ("Identification and Characterization of a Novel Human Matrix Metalloproteinase with Unique Structural Characteristics, Chromosomal Location, and Tissue Distribution," 2012)

Additionally, in MMP 19 the 9-residue insertion, which is rich in hydrophobic amino acids located at the hinge region in stromelysins is replaced by a longer insertion rich in acidic residues. ("Identification and Characterization of a Novel Human Matrix Metalloproteinase with Unique Structural Characteristics, Chromosomal Location, and Tissue Distribution," 2012)

So MMP 19 cannot be classified like the other MMPs, but rather may represent a different new subfamily because of its structural differences.

A study could show that MMP 19 acts anti-angiogenically on endothelial cells due to the generation of three angiostatin-like fragments by cleavage. Those fragments can inhibit capillary-like formation.

Current data show that MMP 19 can act pro- and anti-angiogenic as well as anti-proliferative as secreted active and has an influence on the bioavailability of VEGF and MMP 2. ("BMC Biochemistry | Full text | Matrix metalloproteinase-19 inhibits growth of endothelial cells by generating angiostatin-like fragments from plasminogen," 2012)
It is expressed by endothelial and vascular smooth muscle cells *in vivo* and was found downregulated during histologic dedifferentiation and transformation in the epidermis. ("Matrix metalloproteinase-19 is expressed by pro... [Int J Cancer. 2003] - PubMed - NCBI," 2012)

3.10 Metalloproteinases (MMPs) and invasiveness

These proteolytic enzymes are essential for the invasion, because they enable the migration of the extravillous trophoblast cells into the endometrial stroma.
MMP 2, 7 and 9 are found to be predominantely expressed in different stages of invasion – but always in the invasive phenotype of trophoblasts. But it is assumed, that the expression of MMPs itself may not be a direct indicator for invasiveness, because they may be required for the turnover of self-secreted extracellular matrix.(Kay et al., 2011)
It has been shown that there are no differences in MMP expression in placentas with reduced and normal invasion. Several publications (Bischof et al., 2000a, 2003; Wang et al., 2001; Campbell et all., 2003 Staun-Ram et al., 2004) could not answer the question as to which role the MMPs play in invasion and/or anchorage of the placenta by the turnover of the extracellular matrix. (Kay et al., 2011)

3.11 Oxidative stress of the placenta in diabetes

In pregnancy oxidative and nitrative stress results from oxidative and nitrative species like nitrogen dioxide, nitric oxide, hydrogen peroxide, hydroxyl and superoxide. Those are reactive species, which have effects on cell fate via second messengers and thereby influence placenta development . Tissue damage and cell death can be caused by the release of free radicals, because they can harm DNA, cellular lipids and proteins. strategies in terms of antioxidants administration exist. In healthy pregnancies those defense processes can convert reactive oxygen species into molecular oxygen and water to prevent an overproduction of reactive oxygen. The most significant antioxidants to avoid damages are vitamins C and E, mitochondrial manganese, catalase, gluthatione peroxidase/reductase, reduced gluthatione and cytosolic copper-zink superoxide dismutases. An imbalance between the formation of reactive oxygen species and antioxidants has often been associated with pregnancy pathologies, like diabetes.(Benirschke et al., 2012)(“Diabetology of Pregnancy,” 2012)

The remodeling of uteroplacental arteries and the invasion of the trophoblast cells are processes associated to the mature placental function that can be highly affected by hyperglycemia-induced nitrative and oxidative stress. (“The role of oxidative stress in the pa... [Antioxid Redox Signal. 2011] - PubMed - NCBI,” 2012)

Animal models, like Cohen diabetes sensitive rats, show higher levels of oxidative stress when glucose concentrations are increased. This could be one reason for placental changes under diabetic conditions. Especially superoxide and ketone bodies are enhanced, whereas gluthatione and nicotinamide adenine dinucleotide phosphate (NADPH) are reduced. Thus there is an increase in oxidative and nitrative stress markers in diabetic pregnancies and this may cause an imbalance in stressors over defendors i.e. key players of the antioxidant system.(Kay et al., 2011)

The antioxidant defense systems of diabetic women are altered in the way that the levels of gluthatione reductase and catalase are increased in the placental tissue. Whereas there have not been found changes in the levels of superoxide dismutase and glutathione peroxidase. Compared to nondiabetic placental tissues less response to exogenous oxidative stress, less catalase and a decrease of glutathione peroxidase activity have been there observed in diabetic. More data for alterations in oxidative stress and antioxidative defence are summarized in the following table:

	Pregestational DM	Gestational DM
Antioxidants		
Superoxide dismutase	↓	=/↓
Catalase	-	↑/↓
Gluthatione peroxidase	↓	=/↓
Gluthatione reductase	-	↑
Vitamin E	=/↓	=/↓
Gluthatione	↑	↑
Markers of oxidative and nitrative stress		
Malondialdehyde	↑	↑
Thiobarbituric acid-reactive	-	↑
8-isoprostane	-	=/↑
Xanthine oxidase	-	↑
Nitrites/Nitrates	↑	-

(↓ decrease ↑ increase = no change)

Figure 9 shows alterations of antioxidants and markers of nitrative and oxidative stress in pregestational and gestational diabetes mellitus.

The conclusion of these data is that oxidative and nitrative stress also occurs in healthy pregnancies, but is even more apparent in diabetic pregnancies caused by decreased antioxidant defense mechanisms. (Kay et al., 2011)

3.12 Enzymatic antioxidants

ROS which are produced in the mitochondria, act as second messengers and are meant to maintain the redox homeaostasis for the protection against oxidative stress. ROS overproduction may be induced by proinflammatory mediators (target proteins like MMP-9 for example)and leads to oxidative stress as well as any imbalance between reactive oxygen species and antioxidant defenses.

The accumulation of ROS causes mutations in the DNA which leads to damages in the proteins and lipids. This means oxidative stress for the organism and may lead to pregnancy pathologies like intrauterine growth restriction, miscarriage or preeclampsia. Oxidative stress can be limited by the antioxidant enzymes like Nox5, Sod3 or GPx-3 and the uncoupling proteins (UCPs). ("Antioxidant defenses in the rat placenta in late... [Biol Reprod. 2010] - PubMed - NCBI," 2012)("Role of NADPH oxidase/ROS in pro-inflammat... [Biochem Pharmacol. 2012] - PubMed - NCBI," 2012)

There exist different subtypes of these oxidases which are located in different subcellular areas. There, they produce superoxide which will be converted into hydrogen peroxide. They are regulated by their regulatory subunits and triggered by hormones, cytokines and vasoactive agents. ("NADPH Oxidases: Functions and Pathologies in the Vasculature," 2012)

Low oxygen environment like in early pregnancy is assumed to limit ROS and this may prevent the placenta from damages caused by these oxidative stress. This is an important fact, because the placenta as a tissue of high metabolic activity and cell division is very sensitive. In the late first trimester of pregnancy an increase in antioxidant capacity and antioxidant enzyme activity can be determined.

Alterations in the expression of these antioxidative enzymes have been seen in third trimenon rat placentas, which suggests that this protects the placenta of damages caused by reactive oxygen „at a time when placental vascular adaptions facilitate large increases in oxygen delivery and utilization." The rat placenta showed „highly dynamic and zone-specific patterns of antioxidant gene expression over the final third of gestation." ("Antioxidant defenses in the rat placenta in late... [Biol Reprod. 2010] - PubMed - NCBI," 2012)

Reactive oxygen species also play a role in the immune system to control microbial infections for example-it is part of the effector mechanisms in host defense. It is therefore produced by phagocytes through the activation of NADPH-oxidase complex. So superoxide and other reactive molecules like peroxide and hydroxyl radicals are generated and they can cause the death of the host by damage of their macromolecules. ("Extracellular superoxide dismutase protects hist... [PLoS Pathog. 2012] - PubMed - NCBI," 2012)

During implantation the ability of the trophoblast to invade the uterus is related to production of NO, because it dilates the vessels and turns the uterus receptive to trophoblast penetration, but NO overproduction can also induce trophoblast apoptosis. ("The role of oxidative stress in the pa... [Antioxid Redox Signal. 2011] - PubMed - NCBI," 2012)

3.12.1 NOX5

NOX5 is a member of the NADPH oxidase enzyme family which in the vascular system and comprises 7 members: DUOX 1 and 2 and the NOXs 1-5. These oxidases catalyze the reduction of molecular oxygen, which is NADPH dependent, by moving an electron across cellular membranes, transferring it to oxygen and thereby generate superoxide anion and therefore reactive oxidative species. ("NOX5: from basic biology to signaling an... [Free Radic Biol Med. 2012] - PubMed - NCBI," 2012)

The NOX enzymes play a role in cellular functions like proliferation, apoptosis, differentiation and growth and are involved in pathologies of the vascular system like hypertension, artherosclerosis, restenosis, inflammation, and diabetes". ("NADPH Oxidases: Functions and Pathologies in the Vasculature," 2012)

These vascular noxes are activated by chemical factors like changes in pH and O_2, vasoactive agents like angiotensin II or endothelin-1 and physical factors like streching and pressure. This is regulated by phosphorylation of NADPH oxidase regulatory subunits and *de novo* protein synthesis of NOX homologues. NOX5 possesses an amino-terminal calmodulin-like domain with 4 binding sites for Ca^{2+} and therefore does not require other subunits to get activated. Its regulation at the translational and transcriptional levels happen by Angiotensin II through Ca^{2+}- and calmodulin-sensitive processes.. ("Nicotinamide adenine dinucleotide phosphate reduced... [Circ Res. 2010] - PubMed - NCBI," 2012)

NOX-5 plays an important role in nonspecific host defense, where it was first discovered in neutrophils. It consists of membrane-bound subunits gp91 phox (NOX2)/NOX1/NOX4, p22 phox and the cytoplasmic regulatory components p47 phox and p67 phox. ("The role of oxidative stress in the pa... [Antioxid Redox Signal. 2011] - PubMed - NCBI," 2012)

NOX isoforms could be found expressed in cells emerged from human cancers. Additionally NOX5 is expressed in fetal tissues, adult spleen and uterus. ("Homologs of gp91phox: cloning and tissue expression of ... [Gene. 2001] - PubMed - NCBI," 2012) But although it is found expressed in angiogenesis and endothelial cell proliferation, the functional relevance of vascular NOX5 could not been demonstrated yet.

There have been identified 5 different isoforms of NOX5: NOX5α, NOX5β, NOX5γ and NOX5δ.

Under hypoxic conditions like in diabetes, NADPH oxidase can be stimulated by angiotensin II, AGE and insulin (Figure 9). The NADPH oxidase, once activated in response to high glucose catalyzes the transfer of electrons from NADPH to molecular oxygen to produce O_2^- and H_2O_2. As a result of that, ROS are generated by stimulation of NADPH oxidase in hyperglycaemic conditions. ("The role of oxidative stress in the pa... [Antioxid Redox Signal. 2011] - PubMed - NCBI," 2012)

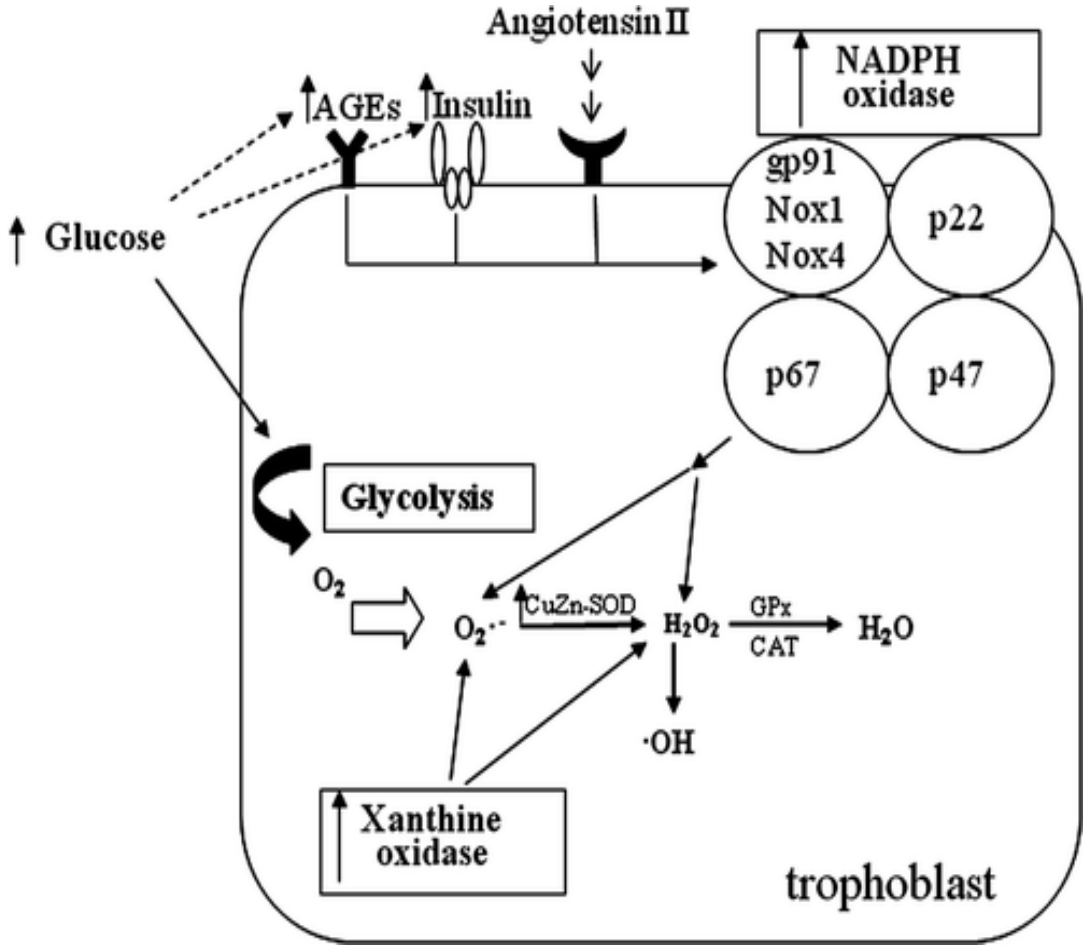

Figure 10 Cytosolic formation of ROS and its degradation

↑ : increased levels of glucose, AGE and insulin in the maternal/fetal circulation or increased activity in the placenta in GDM.

Under physiological conditions the elimination of ROS is achieved by cellular defense mechanisms. If an imbalance of ROS production (in hyperglycemia for example) and antioxidant systems of a cell occurs, this can lead to an upregulation of antioxidant gene expression by activation of nuclear antioxidant response elements (ARE) through the redox-sensitive transcription factor nrf2.

In endothelial cells of pregnant women with gestational diabetes mellitus, NADPH oxidase was shown to be higher expressed and was suggested to represent the major enzymatic source of superoxide in the placenta. (“The role of oxidative stress in the pa... [Antioxid Redox Signal. 2011] - PubMed - NCBI,” 2012)

3.12.2 SOD3

SOD3 is an antioxidative metalloenzyme which belongs to the group of superoxide dismutases. These superoxide dismutases catalyze the conversion of superoxide to hydrogen peroxide (H_2O_2) and oxygen, whereas H_2O_2 is inactivated by gluthatione peroxidase, catalase and the thioredoxin system. ("Antioxidant defenses in the rat placenta in late... [Biol Reprod. 2010] - PubMed - NCBI," 2012) There exist three isoforms of the superoxide dismutases: the cytosolic SOD1, the mitochondrial SOD2 and the extracellular SOD3. ("Effects of labor on placental expression of superox... [Placenta. 2010] - PubMed - NCBI," 2012a)

It can be found expressed in tissues like the lungs, blood vessels, kidneys and also the placenta. ("Effects of labor on placental expression of superox... [Placenta. 2010] - PubMed - NCBI," 2012b)

Extracellular superoxide dismutases like SOD3 are able to oppose or neutralise host-derived ROS and so on play a role in host defense of the organism. ("Extracellular superoxide dismutase protects hist... [PLoS Pathog. 2012] - PubMed - NCBI," 2012)

A study showed that extracellular superoxide dismutases figure into organogenesis of the mouse in the way that these antioxidant enzymes defend the organism against endo- and exogenous oxygen stresses and that they are expressed in extraembryonic tissues and embryos, as well as in the vasculature system and the lung (extracellular superoxide dismutase). ("Expression profiles of extracellu... [Gene Expr Patterns. 2011 Mar-Apr] - PubMed - NCBI," 2012a) ("Extracellular superoxide dismutase prote... [Free Radic Biol Med. 2011] - PubMed - NCBI," n.d.)

3.12.3 GPX-3

Gluthation peroxidase-3 (GPX-3) is an antioxidant enzyme which contains selenocystein and functions as a compensator of ROS in the extracellular compartment. It is able to maintain the antithrombotic and vasorelaxant properties of the endothelium in the vascular system. ("Glutathione peroxidase-3 deficiency promotes pla... [Circulation. 2011] - PubMed - NCBI," 2012)

It plays a role in the regulation of „platelet activity, endothelial function, platelet-dependent thrombosis, and vascular thrombotic propensity." ("Glutathione peroxidase-3 deficiency promotes pla... [Circulation. 2011] - PubMed - NCBI," 2012) Thus it was found deficient and reduced in function in pathologies like thrombosis and stroke.

There exist 5 different GPX isoforms, but GPX-3 is the only one which was found in the extracellular space. It can diminish oxidative stress by the reduction of hydrogen peroxide and hydroperoxides to alcohol. Hydrogen peroxide and hydroperoxides are usually generated by the platelets to reduce the inhibitory effects of NO, which acts antithrombotically on the platelets, and to promote activation of the platelets. As a consequence of that, if these products get reduced by GPX-3 enhanced platelet activation can be prevented and so on pathologies like thrombosis.

A study in which the expression and distribution of the gluthatione peroxidases in normal and preeclampsic pregnancy were measured, showed significant reductions in the expression of these antioxidant enzymes in the placentae from women with preeclampsia. Also the activity and distribution was altered, which may be in relation with the distribution of differential oxygenation regions of the placenta. ("Differential expression and distribution of placent... [Placenta. 2010] - PubMed - NCBI," 2012)

The reactive oxygen species will get enhanced in GPx-3 deficiency and this leads to enhanced oxidative stress to the organism. ("Glutathione peroxidase-3 deficiency promotes pla... [Circulation. 2011] - PubMed - NCBI," 2012)

GPX3 was found to be in a positive correlation with the frequency of leukemia stem cells and so on also with adverse prognostic outcome in human acute myeloid leukemia. ("A role for GPx3 in activity of normal and leukemia... [J Exp Med. 2012] - PubMed - NCBI," 2012)

3.13 MMPs and antioxiant enzymes

Oxidative stress acts as an activator of MMPs by the disruption of the cysteine switch which maintains the latency of proMMPs caused by ROS and NO.

H_2O_2 enhances MMP activity in the fetuses and the maternal side of the placenta from rats, but in contrary to this, SOD and the antioxidant N-acetyl-cysteine (NAC) reduces MMP activity. (“Oxidative stress promotes the increase of mat... [Free Radic Res. 2005] - PubMed - NCBI,” 2012)

Diabetes mellitus also may alter the tissue productions of NO, which regulates the activity of the MMPs. MMP-2 and -9 were found to be increased in the maternal and fetal side of the rat placenta in diabetes, because of higher concentrations of nitrite/nitrate (which indicates production of NO). This may demonstrate the relation between changes in the activation of MMPs and NO and developmental alterations caused by maternal diabetes.(“Increased matrix metalloproteinases 2 and 9 in plac... [Placenta. 2005] - PubMed - NCBI,” 2012) (“The role of nitric oxide on matrix metalloprote... [Reproduction. 2007] - PubMed - NCBI,” 2012)

A study showed, that in the placentae from pre-gestational diabetic patients, in which peroxynitrite-induced damage and NO overproduction has been found, placental MMPs were increased. (“The role of oxidative stress in the pa... [Antioxid Redox Signal. 2011] - PubMed - NCBI,” 2012)

So this suggests, that diabetic conditions can cause changes in the oxidative balance and as a result of that MMPs activity may be increased in the feto-placental unit from diabetic rats.

As a consequence of that associations between oxidative stress and altered development pathways, in which the MMPs are implied may be assumed.(“Oxidative stress promotes the increase of mat... [Free Radic Res. 2005] - PubMed - NCBI,” 2012)

3.14 The housekeeping genes

Housekeeping genes are expressed in every cell of the organism and can act as an intern reference to the expression level of genes we analysed, because they are constitutive and maintain basic cellular functions.

We chose the housekeeping genes RPL 30 and HPRT1. RPL 30, which is 60 S ribosomal protein L30, encodes a ribosomal protein and belongs to the L 30 E family. This ribosomal protein is a component of the 60 S subunit and is located in the cytoplasm.("ScienceDirect.com - Genomics - The mapping of seven intron- containing ribosomal protein genes shows they are unlinked in the human genome," 2012)

HPRT1 stands for the enzyme hypoxanthine-guanine phosphoribosyltransferase, which catalyzes the conversion of guanine to guanine monophosphate and hypoxanthine to inosine monophosphate. This transferase plays a central role in the purine salvage pathway, where it generates purine nucleotides by the transfer of 5-phosphoribosyl from 5-phosphoribosyl 1-pyrophosphate to the purine.

3.15 Research questions and aims

The working hypothesis was that diabetic conditions affect the expression of MMPs and enzymatic antioxidants in human placenta. In order to test this hypothesis I cultivated human placental explants of the first trimenon under different diabetic conditions (different glucose and oxygen concentrations) and subsequently analysed gene expression of MMPs and enzymatic antioxidants.

4 Methods

4.1 Basic experimental setup

Placental explants were incubated in different glucose and oxygen concentrations for 48 hours. Total RNA was isolated and measured to examine the concentration and pureness. Thereafter cDNA out of the RNA was prepared to be used as a template for subsequent RT-PCR analyses.

4.2 Placental tissue

Human first trimester placentas were obtained from elective pregnancy terminations with informed consent of patients. After tissue collection – either by a resident gynaecologist or the department of Obstetrics and Gynecology of the Medical University Graz, placentas were carried in culture medium (DMEM) to the labor by the emergency service. Every placenta has been registered with an anonymous indentification number.

	sample ID	oxygen (%)	glucose (mM)
1	11/055	2,5	25
2	11/055	8	25
3	11/055	12	25
4	11/055	21	25
5	11/055	2,5	16
6	11/055	8	16
7	11/055	12	16
8	11/055	21	16
9	12/003	2,5	25
10	12/003	8	25
11	12/003	12	25
12	12/003	21	25
13	12/003	2,5	16
14	12/003	8	16
15	12/003	12	16
16	12/003	21	16

17	12/004	2,5	25
18	12/004	8	25
19	12/004	12	25
20	12/004	21	25
21	12/004	2,5	16
22	12/004	8	16
23	12/004	12	16
24	12/004	21	16
25	12/011	2,5	25
26	12/011	8	25
27	12/011	12	25
28	12/011	21	25
29	12/011	2,5	16
30	12/011	8	16
31	12/011	12	16
32	12/011	21	16
33	12/012	2,5	25
34	12/012	8	25
35	12/012	12	25
36	12/012	21	25
37	12/012	2,5	16
38	12/012	8	16
39	12/012	12	16
40	12/012	21	16

Table 1 shows the used explants and the conditions in which they have been incubated.

4.3 Explant culture

After placentas had arrived at the institute I transferred the tissue in a culture dish containing PBS buffer.This way I was able to examine by miscroscope wheather the tissue was in good condition and we could use it for our experiments.
Then the well preserved tissues were put in another culture dish, which was filled with pre-warmed DMEM/ Haems F-12 solution (1:1) which contained 1% Penicillin/Streptomycin, 1% Amphotericin B, 10% fetal calf serum and 2mM L-Glutamine. In this solution placental villous tissue was cut with a scalpel into 48 small pieces of approximately 10mg moist mass. We were always working under steril conditions, wearing a laboratory coat and gloves. We also desinfected our hands, working top, materials and equipment with a solution of 75% alcohol. We worked in a steril workbench with current air flow.
I used four 12-well plates for each placenta and every well plate was considered for a defined concentration of oxygen.
In three wells of the 12-well plate I put 2ml of the culture medium including a low glucose concentration (16mM) and 5ml culture medium with high a glucose concentration (25mM) in another three wells.
After that I transferred two pieces of the dissected villi in each well of the well plate.
Then the well plates were placed in different incubators, which were set to the following oxygen concentrations: 2.5% ,8%, 12% and 21%.
The placental explants were incubated for 48 hours.

Figure 11 shows the villi of a a first trimester placenta after elective pregnancy termination.

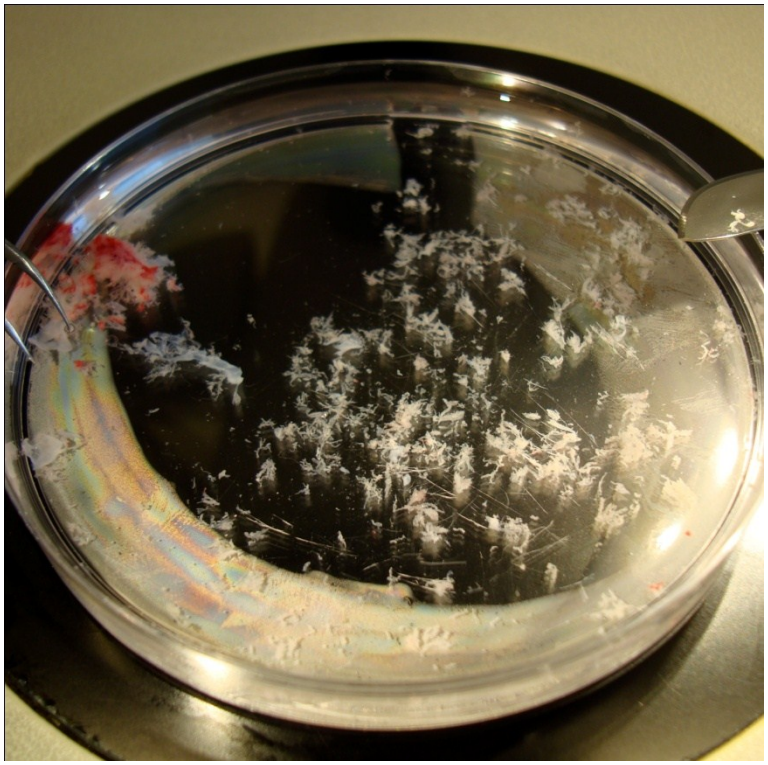

Figure 12 shows the villi after the dissection of the villous tissue.

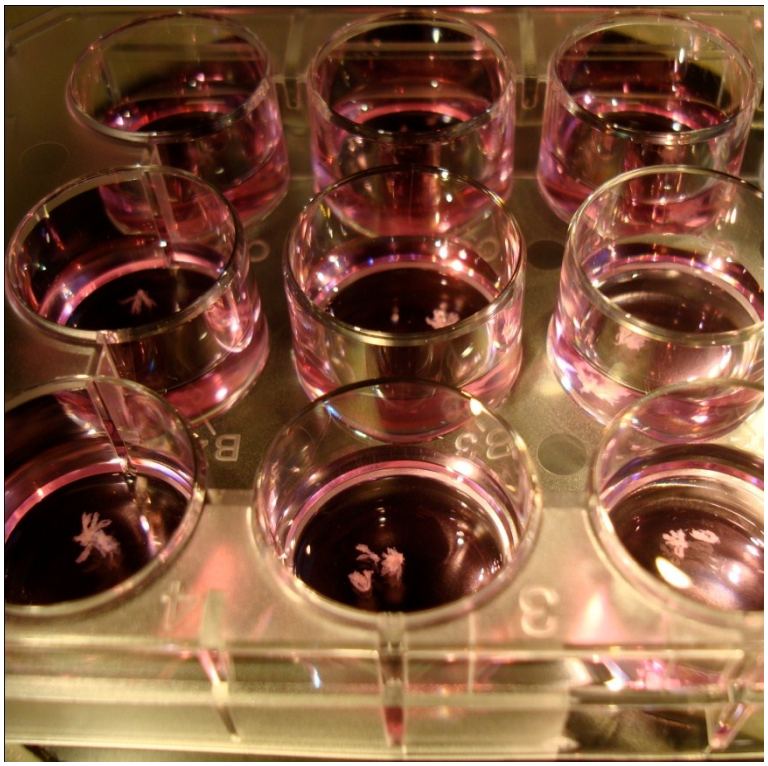

Figure 13 shows the placental explants in a well plate.

4.4 Tissue homogenization

After incubation, the explants were collected from the well plates and were transferred into Eppendorf tubes – explants incubated under identical conditions were pooled in one tube. In total, 8 Eppendorf tubes (i.e. incubation conditions per placenta) were processed. Tubes were filled with 500 µl of Trizol and explants were added to the reagent. Thereafter, the explants in tubes with Trizol were mixed using a tissue homogenizer until placental villi were completely homogenized. Trizol, a reagent commonly used for RNA isolation, is a substance containing guanidiniumthiocyanat and can hereby inactivate RNases and other enzymes. It also contains the reagent phenol in which nucleic acids and proteins can get dissolved. This method is called the single step method and consists of the following steps: First the cells get lyzed, then the different phases get seperated and thereafter the RNA can be precipitated-but this is already a part of the RNA isolation procedure. (Joppien et al., 2010)

The samples were stored in the freezer at -80 ˚C.

4.5 RNA isolation

The isolation of RNA basically consists of two steps: Primarily the cells have to get lyzed to set free their contents. Thereafter the RNA has to be seperated from the other contents of the cell. At this step it is important to eliminate the ribonucleases, which are enzymes who can destroy RNA and occur in almost every cell.

The samples were taken out of the freezer and after defrosting I gave 100µl of BCP to the solution, in which the explants were arranged to seperate the organic phase from the aqueous phase, where the nucleic acids were located.

After shaking I waited for 15 minutes and then I centrifuged it for another 15 minutes at 13 000 g and 4°C.

The supernatand, which could be seen easily after the centrifugation was now pipetted in new Eppendorf tubes, thereby I had to attend to not take the pellet out, too, which was at the bottom of the tube.

In the supernatand we expected to find the RNA containing aqueous phase of the sample. In addition to that I gave 1000µl Isopropanol to the supernatand, because it causes the RNA to precipitate from the aqueous phase.

This is called a alcoholic precipitation, which happens if you give a monovalent salt and alcohol to the nucleic acid.(Mülhardt, 2008)

I shaked the tubes and waited 10 minutes. After another centrifugation, which lasted 8 minutes at 12 000 g, I poured out the supernatand, whereat I payed attention not to lose the pellet at the bottom of the tube.

I added 1ml of cold Ethanol to clean the pellet and purify it from its residues of salt and alcohol. Then I centrifuged for 5 minutes at 7000 g. Afterwards I poured out the Ethanol, again paying attention not to lose the pellet. I tried to pipet of the residual liquids and let the pelett air dry.

Then I put 30µl RNase-free water to the dried pelett and gave the tubes into the thermal shaker for 5 minutes at 55°C, stirred the samples and put them back into the thermal shaker for another 5 minutes.

After the isolation the tubes were chilled on ice for the following measurement of the RNA.

4.6 Messurement of the RNA

The concentration and pureness of the isolated RNA was measured by the spectral photometer NanoDrop ND-1000 from PEQLAB. Those measurements are based on the principle of spectralanalysis of absorptions, which means that the absorption of short-wave light of the used solutions is measured and thereout the concentration can be calculated through the law of Lambeert-Beer. Furthermore the pureness of the RNA can be assumed by building the quotient of the absorptions at 260nm and 280nm. Values around two reflect a relatively pure RNA, whereas smaller values are caused by contaminations with proteins or phenols.("NanoDrop Products - Spectrophotometers and Fluorospectrometers - www.nanodrop.com," 2012)

The measurements were done in the following way: Initially I measured 1.5 µl of Aqua-dest to set a blank value. After removing the water, I did the measurement of every single sample- 1.5µl of each. Between every measurement the device was cleaned with Aqua-dest to prevent inaccuracies.

The results could then be shown on the computer in graphics:

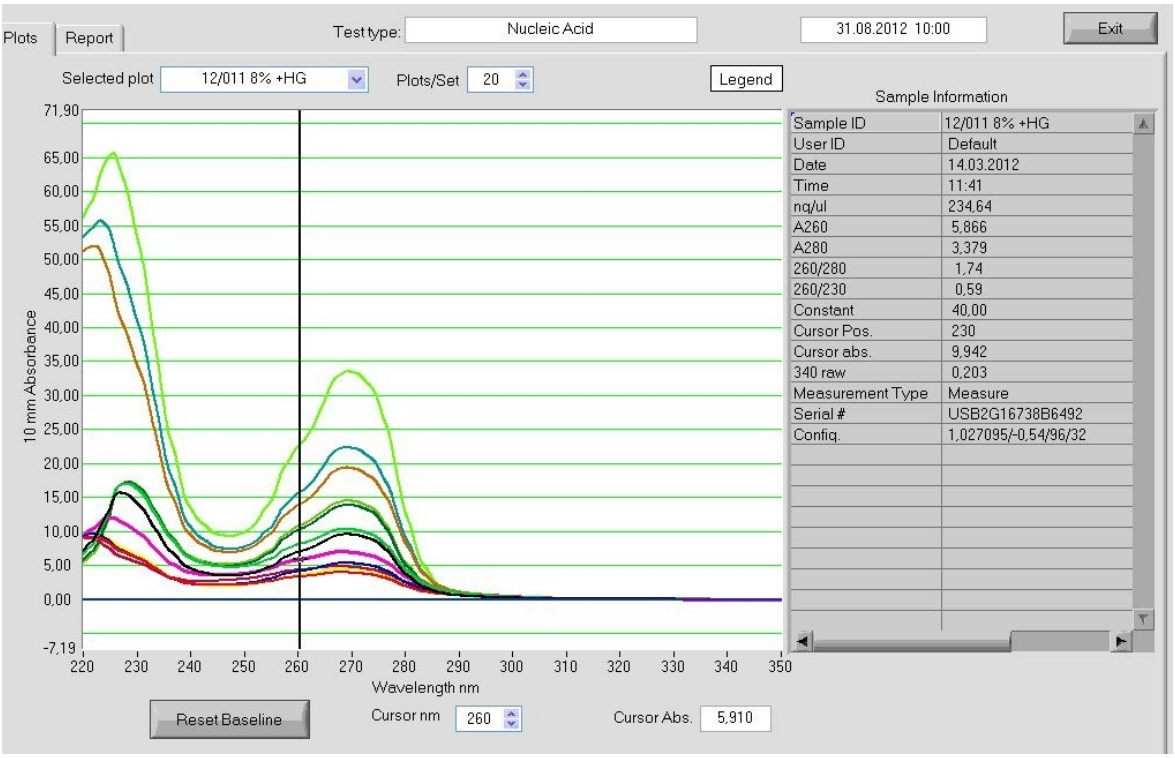

Figure 14 shows the results of the spectralanalysis of the sample 12/011 as an example. The 260/280 ratio of the sample (absorbance at 260 and 280 nm) is used to assess the purity of the RNA. A ratio of ~2.0 is generally accepted as "pure" for RNA. ("NanoDrop Products - Spectrophotometers and Fluorospectrometers - www.nanodrop.com," 2012)

Sample ID	ng/ul	A260	A280	260/280	260/230	Constant
11/048 21% A	791.17	19.779	10.425	1.9	1.11	40
11/048 21% B	938.36	23.459	12.281	1.91	0.79	40
11/048 21% C	702.92	17.573	9.298	1.89	0.96	40
11/048 12% A	920.72	23.018	11.755	1.96	0.93	40
11/048 12% B	765.04	19.126	9.972	1.92	1.17	40
11/048 12% C	627.53	15.688	8.313	1.89	0.85	40
11/048 8% A	958.76	23.969	12.821	1.87	0.59	40
11/048 8% B	463.29	11.582	6.023	1.92	0.86	40
11/048 8% C	752.39	18.81	9.921	1.9	0.95	40
11/048 2,5% A	617.72	15.443	8.155	1.89	1.07	40
11/048 2,5% B	546.78	13.67	7.287	1.88	0.89	40
11/048 2,5% C	786.98	19.674	10.932	1.8	0.6	40

Figure 15 shows the results of the spectralanalysis of the sample 11/048 as an example. The 260/280 ratio of the sample is about 1.8 to 1.96.

4.7 cDNA

The term cDNA stands for complementary DNA, which can be synthesized out of RNA by the enzyme reverse transcriptase. This transcriptase is highly specific and RNA depending. For the synthesis the enzyme needs a short, complementary strand, which is called the primer and binds to the RNA. The produnct of the synthesis is a single strand of cDNA, which is hybridized with the initial RNA strand and can be degraded with an enzyme called RNase H. The RNase H. is a endonuclease which „catalyzes the cleavage of RNA via a hydrolytic mechanism“. (“RNase H - Wikipedia, the free encyclopedia,” 2012) Thereafter the complementary cDNA strand is synthesized to the initial single strand of cDNA by a DNA-depending polymerase. The result is a double-stranded cDNA, which can afterwards be used for the RT-PCR (therefore is this analysis reffered to as RT-PCR)/
For this process we used SuperScript II Transcriptase, which is an engineered version of M-MLV RT with reduced RNase H activity and increased thermal stability. It is purified to near homogeneity from E.coli containing the modified pol gene of Moloney Murine Leukemia Virus. (“superscriptII_pps.pdf,” n.d.)
I added 1µl of Oligo(dT) primers and 1µl of dNTPMix (10mM each) and sterile, distilled water up to 12µl to a nuclease-free microcentrifuge tube. Afterwards I heated the mixture to 65°C for 5 minutes and quick chilled it on ice. Then I collected the contents of the tube by brief centrifugation and added 5X First-Strand Buffer (4µl), 0,1M DTT (2µl) and 1µl of the RNase inhibitor „RNase OUT“ (40units per µl).
I mixed the contents of the tube gently and incubated for another 2 minutes at 25°C. After that I added 1µl (200units) of SuperScript II RT and mixed by pipetting up and down gently. I incubated it for 50 minutes at 42°C and then I inactivated the reaction by heating at 70°C for 15 minutes.(“Invitrogen - Perfect Primer Design with Invitrogen’s OligoPerfect^{TM} Designer,” 2012)
Afterwards the cDNA was diluted up to 200µl, so we added 180µl of Aqua-dest.
The cDNA can afterwards be used as a template for amplification by PCR. This is called real time, two step PCR, because the reverse transcription and the PCR are prepared seperate.

Figure 16 shows the basic principle of two-step PCR.

4.8 Quantitative real time PCR

The basic idea is the examination of the level of regulation of gene expression. The genomic DNA is converted into mRNA, which finally serves as a „construction manual“ for the production of proteins. In this task the RT-PCR is a valid method to analyse the different expression levels of the examined primers/genes. The RT-PCR is a combination of reverse transcription and a following reproduction by PCR.

The RT-PCR, which is a quantitative PCR permits the determination of the relative number of certain cDNA strands in the samples. As there is a mesurement after each single cycle, it is possible to determine the relative amount of new built DNA strands in every single cycle.(Mülhardt, 2008) In consequence of that, you can obtain the number of utilized cDNA and thereby mRNA, too. The number of amplificated products is the result of the determination of the fluorescence after every cycle. This fluorescence increases with increasing amplification and can be measured as a signal at the end of each cycle.(Joppien et al., 2010)

We used fluorogenic-labeled probes, which use the 5’ nuclease activity of Taq DNA polymerase and can increase the specificity of the assays. The TaqMan probes contain a reporter dye, which is linked to the 5’ end of the probe, and a nonfluorescent quencher, which is bound to the 3’ end of the probe. In an intact probe, the fluorescence emitted by the reporter dye is reduced by the proximity of the quencher. This is caused by fluorescence resonance energy transfer which happens through space. But with a target sequence, the probe anneals between the primer sites and gets cleaved by the 5’ nuclease during extension. So the reporter dye gets seperated from the quencher and thereby the reporter dye signal increases.

Additionally this cleavage of the probe causes the removal of the probe from the target strand and as a result of that the primer extension continues to the end of the template strand. With each cycle, reporter dye molecules are seperated from their respective probes. The result is an increase in fluorescence intensity, which is proportional to the amount of produced amplification products.

The TaqMan probes also contain a minor groove binder (MGB) at the 3’ end of the probe. This MGB increases the melting temperature and thereby allows the design of shorter probes. If the starting copy number of the nucleic target is high, a significant increase in fluorescence is observed soon. (“Applied Biosystems,” n.d.)

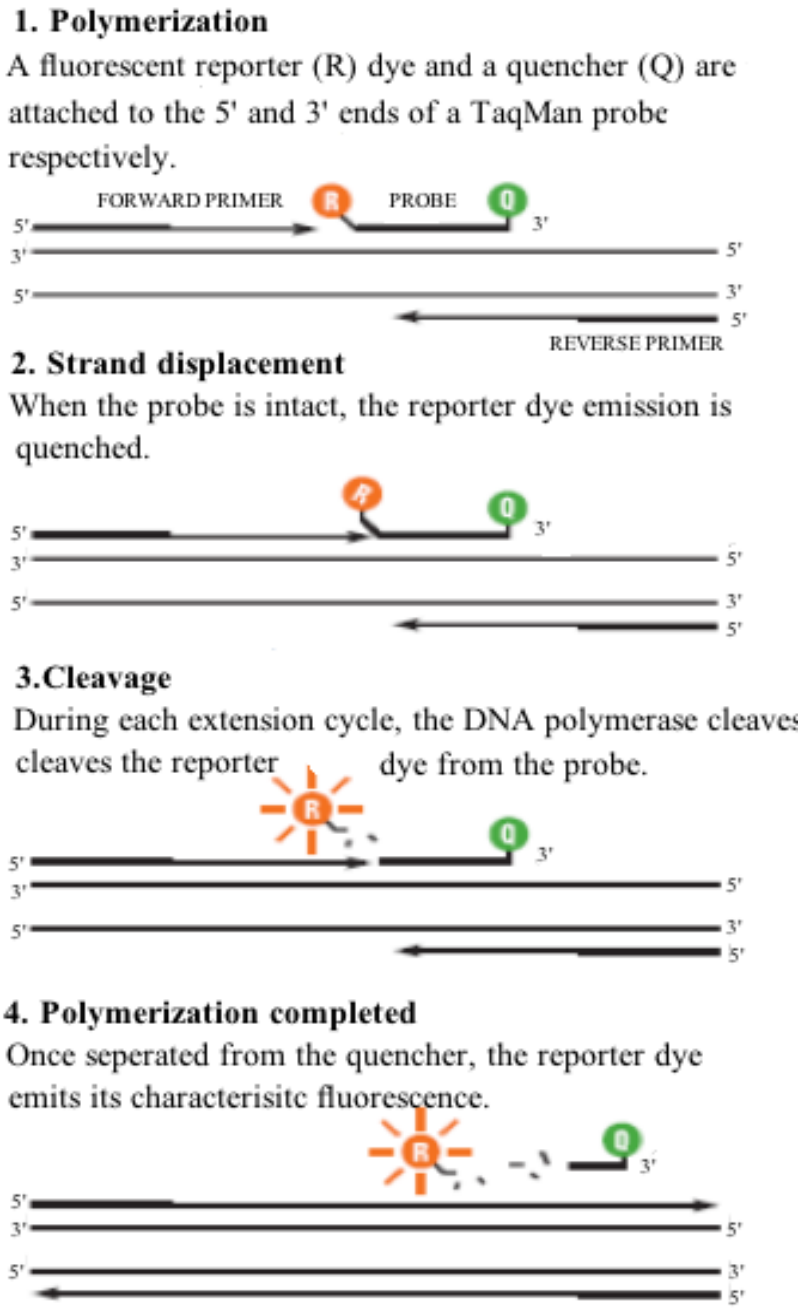

Figure 17 shows the different steps of the TaqMan probe-based assay chemistry.

We used the following primers for MMP-2, 7, 9, 19 and SOD3, NOX5, GPX3, RPL30 and HPRT1 gene expression analysis.

MMP2	Hs01548727_m1
MMP7	Hs01042796_m1
MMP9	Hs00234579_m1
MMP19	Hs00275699_m1
SOD3	Hs00162090_m1
NOX5	Hs00225846_m1
GPX3	Hs00173566_m1
RPL30	Hs00265497_m1
HPRT1	Hs01003267_m1

Table 2 shows the different primers we used and their assay numbers. (Applied Biosystems)

The RT-PCR for each primer performed in the following way:
I put the Mastermix (990µl), RNAse free water (396µl) and the primer (99µl) together in a Eppendorf tube and shaked. The Mastermix includes the HotStarTaq Plus DNA Polymerase, a reporter dye, a nonfluorescent quencher and a minor groove binder.
I labeled 40 small Eppendorff tubes with the numbers from 1 to 40- one for each different cDNA and then pipetted 33µl of the prepared PCR-mastermix into the tubes. To this solution I added 11µl of the different cDNA samples. The tubes were shaked and zentrifuged. Afterwards the mastermixes including a cDNA were transferred into 96-well PCR plates- 20µl in each well, so that every Eppendorf tube was split into two wells of the PCR plates (samples were analyzed in duplicates). The PCR plates were sealed with a sealing foil.
During all of these steps the samples were cooled by ice blocks.
After short centrifugation (600rpm, 1min) the plates were given into the PCR amplification machine, which was programmed to perform the following programm: First the reverse transkription happens at 50°C for 30 minutes. Thereby the primers specifically attach to the complementary nucleotide sequences of the mRNA and the reverse transcriptases begin to produce cDNA in 5'-3'direction by consumiption of the dNTPs.
The cDNA which was developed in that way is like a mirror image of the mRNA, which means that there exist no introns in it. After that the HotStar Taq DNA polymerase is

activated at 95°C and thereby the reverse transcriptases get deactivated. The HotStarTaq DNA-polymerase is a thermostable polymerase, which is produced in a recombinant way in E.coli stocks. This polymerase is inactivated by covalent bonding at lower temperatures and only avticated by destroying these bodings at 95°C. This initial PCR activation step takes 10 minutes. Furthermore the hydrogen bridge linkages between the nucleic acids get dissolfed by those high temperatures in this step, so that those linkages will be unpaired and denaturated afterwards.("QIAGEN - QIAGEN Literature and Protocols," 2012) Following a 2-step cycling, the DNA was getting denaturated (15 sec at 95°C) to seperate the associated DNA strands, annealed for hybridization and extended (both for 1 minute at 60°C). For the extension the polymerase starts at the 3'end of the primer and fills up the missing nucleotides so that there emerged a long strand.of DNA. The final extension took about 10 minutes at 72°C.

step	time	temperature
PCR initial activation step	10 min	95°C
Denaturation	15 sec	95°C
Combined annealing/extension	1 min	60°C

Table 3 gives an overview of the different steps of the RT-PCR.

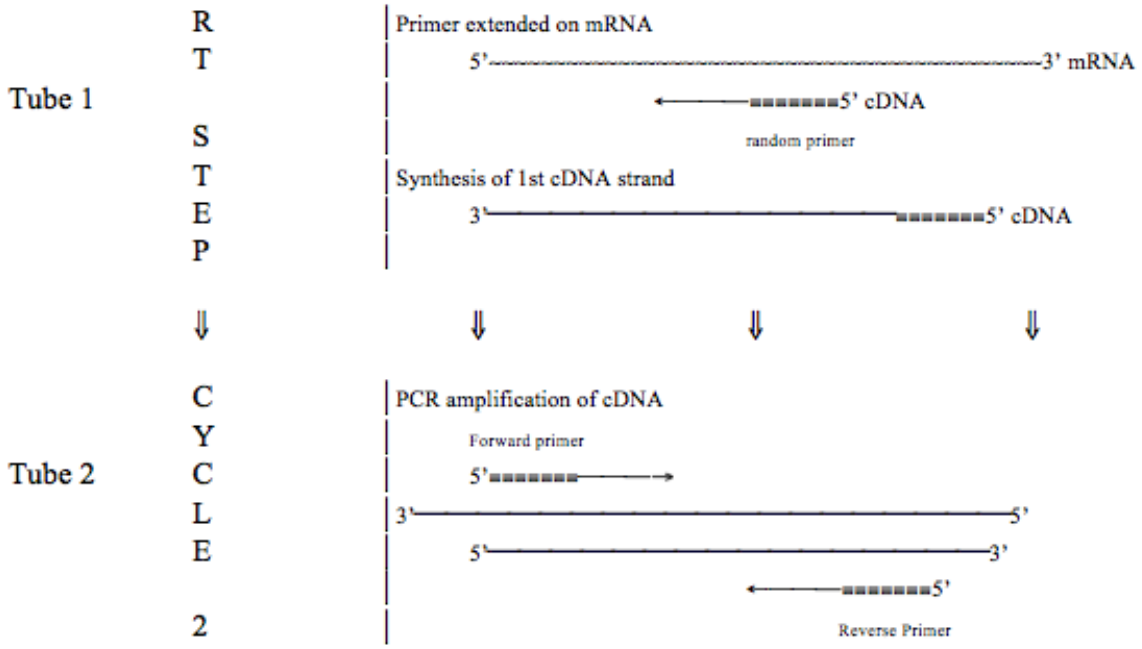

Figure 18 shows the two reactions of the two step real time PCR. In tube 1 the reverse transcription is performed followed by step two, which consists of the PCR amplification.

The steps, denaturation, annealing and elongation were programmed to be repeated for 40 times. After about 4 to 5 cycles especially the required sequences increase exponentially, whose endings and length was determined before by the chosen forward- and reverse-pairs of primers.The amplification duplicates the number of copies every cycle (by the power of 2 number of cycles)-in reality however the factor is about 1,7. At the beginning the graph which expresses the amplification increases exponentially, but near the end it arrives a phase of plateau, because the substrates get consumed and the enzymes lose ist effectiveness through the inhibiting effect of the accumulation of products like pyrophosphate and through the high temparatures. It happens that there occur unspecific and involuntary annealinig of the amplification products with increasing frequency, because there exist fewer and fewer primers and evermore amplification products. ("Critical_Factors_for_Successful_Real-Time_PCR.pdf," n.d.) In this process it is important to have the same efficiency in the PCR reactions.

5 Material

clear solution	gibco®, life technologies
DMEM/Haems F-12	gibco®, life technologies
Trizol	TRIzol®, Molecular Research Center (MRC)
BCP	Molecular Research Center (MRC), Inc.: USA
Isopropanol	Merck KGaA, Germany
Ethanol	Merck KGaA, Germany
RNase-free water	QIAGEN, GmbH, Hilden Germany
Oligo (dT)	invitrogen™, life technologies™
dNTP Mix	QIAGEN, GmbH Germany
5X First-Strand Buffer	QIAGEN, GmbH Germany
DTT	invitrogen™, life technologies
Rnase OUT	invitrogen™, life technologies
SuperScript II RT	invitrogen™, life technologies™
Mastermix	QIAGEN, GmbH Germany
SuperScript II Transcriptase	invitrogen™, life technologies™

Table 4: list of solutions

Petri dish	NUNC™, Denmark
scalpel	Braun, Aesculap division
workbench	Thermo Scientific Germany
well plates	NUNC™, Denmark
incubator	BioSpherix, Xvivo System, model G300C
Eppendorf tubes	Eppendorf Austria
freezer (-80 °C)	Liebherr, Comfort, Austria
centrifuge	WEALTEC E-Centrifuge,Laborfuge 400R Heraeus, function line
Pipettes	Sterilin® Limited, UK
Pipette tips	Eppendorf Austria
thermal cycler	BIO RAD DNA Engine Dyad Peltier Thermal Cycler
sectral photometer	PEQLAB Biotechnologie GmbH Nanodrop ND-1000
PCR system	GeneAmp PCR System 9700, Appied Biosystems QuantiFast SYBR Green PCR Handbook
PCR film	American Natin cal Can.™, USA

Table 5: list of tools

6 Results

6.1 Evaluation of the RT-PCR results

The typical process of a RT-PCR shows a S-figured graph. The significant element to analyse this graphics is the so called threshold cycle (Ct), which reflects the cycle in which the graph crosses a first defined threshold shortly after the signal of the fluorescence starts to raise notably above the basisline (which reflects the background fluorescence). Accordingly to that there has to be set a threshold value to get applicable Ct-values which therefore has to be in the exponential, increasing part of the graph. As earlier as the graph of a sample crosses the threshold-line, as smaller is the Ct-value und accordingly to it contains more cDNA and consequently the sample contained more mRNA.("Tools," 2012) We evaluated our results by relativ quantification with the housekeeping genes RPL30 and HPRT1, which can be seen as reference because they are not regulated and have the same expression in every cell.

We used the ΔΔCt method as a relative quantification method and Microsoft Excel to evaluate the RT-PCR results. The term relative quantification means that the amount of target will be compared to a reference nucleic acid, the housekeeping gene. So it is possible to compare the different gene expression in the samples and calculate the ratio between the target and the reference gene.("QIAGEN - QIAGEN Literature and Protocols," 2012) The housekeeping genes act as an intern control to compensate variations of the used RNA-values.("Real time quantitative PCR – Wikipedia," 2012) The different expressions of the examined genes will get stated with the help of the Ct-value. First of all the Ct values of the examined target genes will get substracted from the housekeeping gene - this determines the ΔCt-value of each sample. Thereafter the ΔCt-values of the samples under different conditions (glucose and O2) can get compared by the ΔΔCt-value. This ΔΔCt can then get plugged in the equation: sample A (e.g. diabetic) compared to sample B (control) = $2^{-\Delta\Delta Ct}$. The threshold was chosen automatically by the program itself and filed the graphs in their exponential phase. The exponential phase is focused in the PCR, because it provides the most accurate and precise data for the quantification.(29)

The Ct-values were taken over to Excel and the mean values were calculated from duplicates.

With the Ct values it is possible to calculate the starting amount of the templates in each sample, so those are the cycles in which the first detectable increase in fluorescence can be detected. Thereafter the Ct-values can be produced „as sigmoidal-shaped amplification plots, in which fluorescence is plottet against the number of cycles“(“QIAGEN - QIAGEN Literature and Protocols,” 2012).

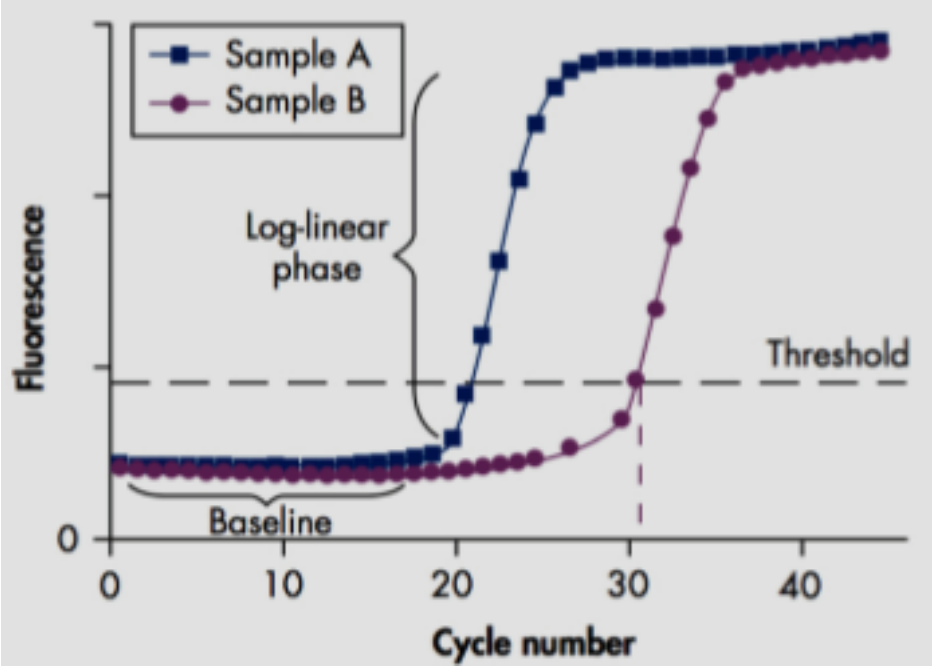

Figure 19 shows the amplification plots which indicate the increases in fluorescence from sample A and B. Sample B contains a lower amount of starting template than sample B.

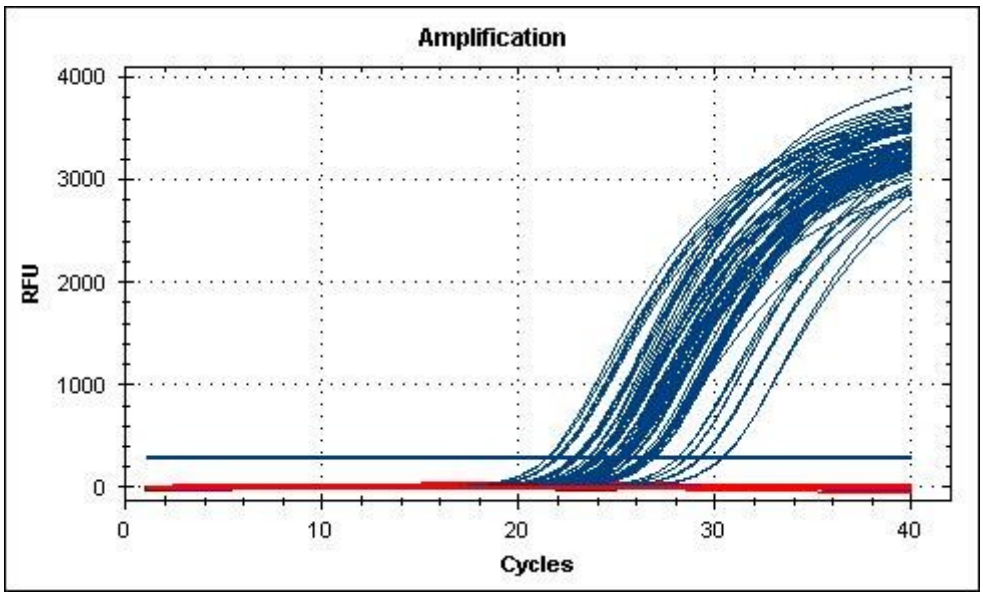

Figure 20: amplification plots from MMP2 analysis (as an example)

6.2 Tests of normal distribution and choice of utilized testing procedure

The statistical evaluation was performed by SPSS for windows, version 19.0 (SPSS Inc., USA). The demonstration of the metrical variables happened by arythmetic averages and medians, whereas the spreads were indicated by standard deviations and quartiles. The metrical variables were reviewed in regard to their normal distribution by use of the Shapiro-Wilk-Test. Some of the tested variables did not have a normal distribution (Shapiro-Wilk-Test: $p<0.05$), but for ΔCt of MMP-2, -9 and GPx-3 there could be calculated a normal distribution (Shapiro-Wilk-Test: $p \geq 0.05$).

We used tests for normal distributed samples and non-parametrical tests for samples which were not normal distributed.

The t-test was used to compare 2 independent, normal distributed samples. But previous to this t-test, the Mann-Lavene-Test was utilized to verify the homogeneity of the variances. For normal distributed samples the Mann-Whitney-U-Test as a non-parametrical method was applied.

We used the H-Test of Kruskal and Wallis to compare more than 2 independent, not normal distributed samples, whereas for the comparison of more than 2 independent, normal-distributed samples the single factor variance analysis ONEWAY ANOVA was applied.

There was made a bipartite significance-verification for every performed test, in which a p-value < 0,05 was considered as significant for every statistical Test.

We used error bars in the graphical representations, which were also created with SPSS, to demonstrate the arithmetical averages at the normal distributed samples. In this connection the standart errors were listed as measure of variation, because of the high scattering of the results.

To present the medians and interquartile ranges of the samples, which were not normal distributed, we utilized boxplots. The median and the 25^{th}-75^{th} percentile are shown in the boxes, whereas the smallest and highest values correspond to the T-bars in case they are not an extreme value or outlier. The values which are considered to be outliers are values which lie between 1 ½ and 3 box-lengths outside of the box. These outlier values are presented by the circles in the graphics, whereas the extreme values, which were measured as more than 3 box-lengths outside of the box, are presented by crosses.

	df	Significance
Delta Ct SOD3	39	**.011**
Delta Ct GPX3	39	**.155**
Delta Ct NOX5	39	**.002**
Delta Ct MMP2	39	**.194**
Delta Ct MMP7	39	**.024**
Delta Ct MMP9	39	**.761**
Delta Ct MMP19	39	**.003**

Table 6 shows the tests for normal distribution (Shapiro-Wilk-Test). The tested variables MMP-7, -19, NOX3 and SOD3 did not have a normal distribution (Shapiro-Wilk-Test: $p<0.05$), but for the Δ CTs of MMP-2, -9 and GPx-3 there could be calculated a normal distribution (Shapiro-Wilk-Test: $p \geq 0.05$). This significance is highlighted by the red colour of the results.

We also used a variant of the Kolmogorov Smirnof-Test, which is called Lilleforts-Test to correct the significance.

6.3 Comparison of Delta Ct concerning oxygen and glucose

6.3.1 Δ Ct GPX3

Graphical overview

The expression of GPX3 under different concentrations of oxygen and glucose did not show a significant variance.

For the group of low glucose (16mM), there may be an increasing trend from 2.5% to 8.0% of oxygen, but in contrary to that, in the range of 8.0% to 21,0% there may be a decreasing trend of the ΔCt values with increasing oxygen concentration. This decrease of the ΔCt of the low glucose group reflects a higher expression of the gene, so GPX-3 in a glucose concentration of 16mM may be higher expressed from 8.0% to 21.0% of oxygen.

This trend for increasing ΔCt values from 2.5% to 8.0% oxygen and decreasing trend from 8% to 21% may also be observed in the group of high glucose (25mM).

In conclusion, the expression of GPX-3 may be expressed higher in increasing concentrations of oxygen.

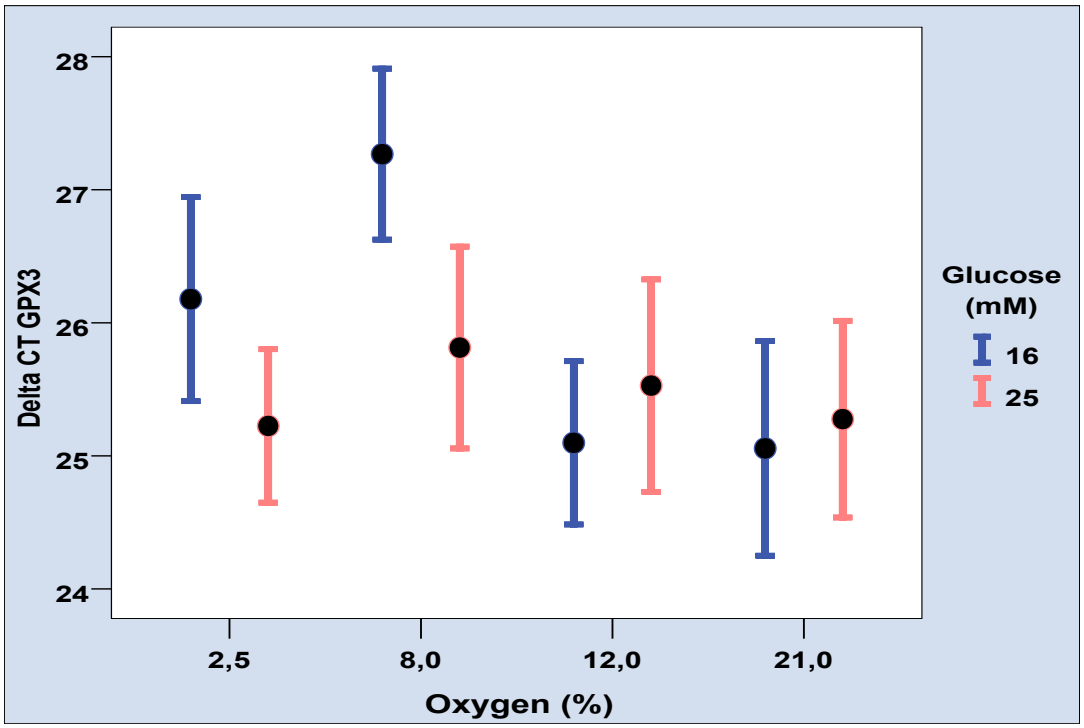

Figure 21 shows the Delta Cts of GPX3 in different glucose and oxygen concentrations.

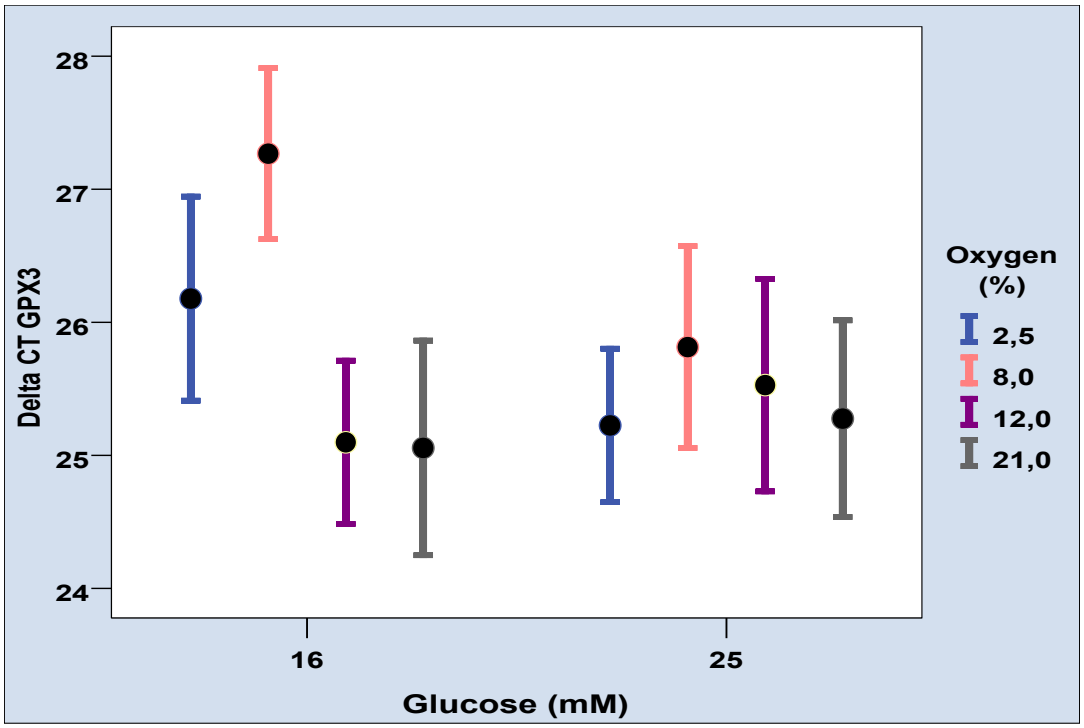

Figure 22 shows the Delta Cts of GPX-3 in different glucose and oxygen concentrations. In an 2.5% and 8.0% oxygen concentration, the Delta Cts of GPX-3 showed higher values in 16mM glucose than in 25mM glucose, whereas in an 12.0% and 21.0% oxygen concentration, the Delta Cts showed higher values in the higher glucose concentration.

6.3.1.1 Effect of oxygen on GPX-3 expression

Results for glucose 16mM

Oxygen (%)	Mean value	Standard-deviation	Standard-error of mean value	Median	Minimum	Maximum	N
2,5	26.1780	1.71479	.76688	26.8100	23.97	27.95	5
8,0	27.2680	1.43637	.64237	27.4400	25.20	28.99	5
12,0	25.0980	1.37190	.61353	25.4700	23.35	26.89	5
21,0	25.0560	1.80359	.80659	25.3200	23.10	26.99	5
In total	25.9000	1.73321	.38756	26.0900	23.10	28.99	20

Table 7 shows the Delta Cts of GPX3 in different oxygen concentrations and the glucose concentration of 16mM.

	Significance
Between the groups In total	**.131**

Table 8 shows the result of the ONEWAY ANOVA of the Cts of GPX3 in different oxygen concentrations and the glucose concentration of 16mM, which is not significant (p=0.13).

Results for glucose 25mM

Oxygen (%)	Mean value	Standard-deviation	Standard-error of mean value	Median	Minimum	Maximum	N
2,5	25.2250	1.15252	.57626	25.2150	23.83	26.64	4
8,0	25.8140	1.69674	.75881	25.2500	23.92	27.87	5
12,0	25.5280	1.78524	.79838	25.0000	24.35	28.68	5
21,0	25.2760	1.65314	.73931	24.8700	23.20	26.97	5
In total	25.4732	1.49475	.34292	25.0300	23.20	28.68	19

Table 9 shows the Delta Cts of GPX3 in different oxygen concentrations and the glucose concentration of 25mM.

	Significance
Between the groups In total	**.939**

Table 10 shows the result of the ONEWAY ANOVA of the Cts of GPX3 in different oxygen concentrations and the glucose concentration of 25mM, which is not significant (p=0.94).

6.3.1.2 Effects of glucose on GPX-3 expression

Results for oxygen 2.5%

Glucose (mM)	Mean value	Standard-deviation	Standard-error of mean value	Median	Minimum	Maximum	N
16	26.1780	1.71479	.76688	26.8100	23.97	27.95	5
25	25.2250	1.15252	.57626	25.2150	23.83	26.64	4
In total	25.7544	1.49019	.49673	25.3500	23.83	27.95	9

Table 11 shows the result of the Delta Cts of GPX3 in different glucose concentrations and the oxygen concentration of 2.5%. The result of the t-Test did not show a significant variance (p=0.37).

Results for oxygen 8.0%

Glucose (mM)	Mean value	Standard-deviation	Standard-error of mean value	Median	Minimum	Maximum	N
16	27.2680	1.43637	.64237	27.4400	25.20	28.99	5
25	25.8140	1.69674	.75881	25.2500	23.92	27.87	5
In total	26.5410	1.66846	.52761	26.9800	23.92	28.99	10

Table 12 shows the result of the Delta Cts of GPX3 in different glucose concentrations and the oxygen concentration of 8.0%. The result of the t-Test did not show a significant variance (p=0.18).

Results for oxygen 12.0%

Glucose (mM)	Mean value	Standard-deviation	Standard-error of mean value	Median	Minimum	Maximum	N
16	25.0980	1.37190	.61353	25.4700	23.35	26.89	5
25	25.5280	1.78524	.79838	25.0000	24.35	28.68	5
In total	25.3130	1.51800	.48003	25.0150	23.35	28.68	10

Table 13 shows the result of the Delta Cts of GPX3 in different glucose concentrations and the oxygen concentration of 12.0%. The result of the t-Test did not show a significant variance (p=0.68).

Results for oxygen 21.0%

Glucose (mM)	Mean value	Standard-deviation	Standard-error of mean value	Median	Minimum	Maximum	N
16	25.0560	1.80359	.80659	25.3200	23.10	26.99	5
25	25.2760	1.65314	.73931	24.8700	23.20	26.97	5
In total	25.1660	1.63518	.51709	25.0950	23.10	26.99	10

Table 14 shows the result of the Delta Cts of GPX3 in different glucose concentrations and the oxygen concentration of 21.0%. The result of the t-Test did not show a significant variance (p=0.85).

6.3.2 Δ Ct NOX5

Graphical overview

The expression of NOX-5 under different concentrations of oxygen and glucose did not show a significant variance.

For the group of low glucose (16mM), there may be an increasing trend of the medians from 2.5% to 8.0% of oxygen, but in contrary to that, in the range of 8.0% to 21.0% there may be a decreasing trend of the ΔCt values with increasing oxygen concentration. This decrease of the ΔCt of the low glucose group reflects a higher expression of the gene, so NOX-5 in a glucose concentration of 16mM may be higher expressed from 8,0% to 21,0% of oxygen.

In the group of high glucose (25mM) the medians show a slight decreasing trend with increasing oxygen concentration, which may reflect a higher expression of NOX-5 in increasing oxygen concentrations.

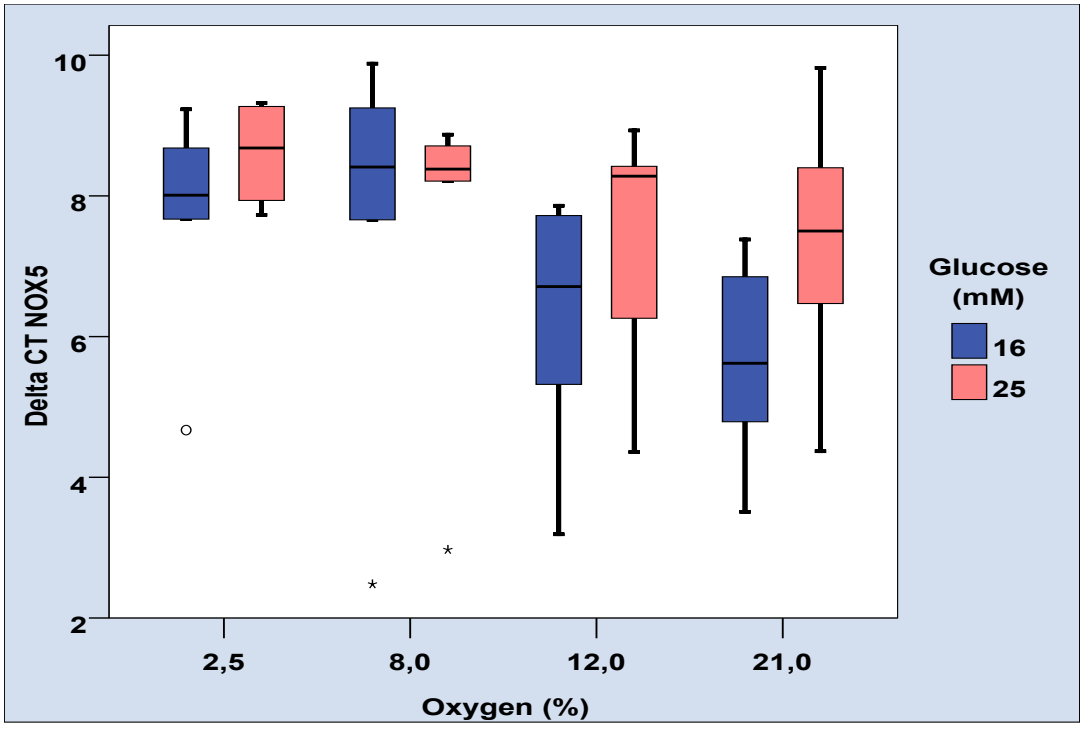

Figure 23 shows the Delta Ct values of NOX5 in different glucose and oxygen concentrations.

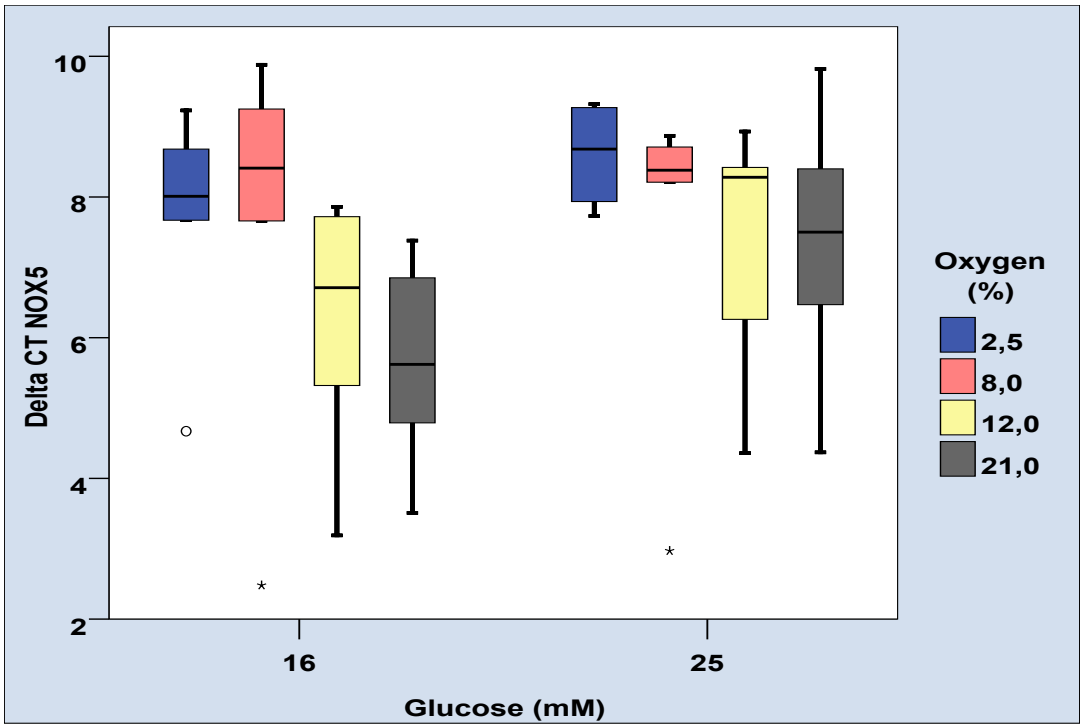

Figure 24 shows the Delta Ct values of NOX5 in different glucose and oxygen concentrations. In an 2.5%, 12.0% and 21.0% oxygen concentration, the medians of the Delta Cts of GPX-3 showed lower values in 16mM glucose than in 25mM glucose, whereas in an 8.0 oxygen concentration, the medians did not show a notable difference in the different concentrations of glucose.

6.3.2.1 Effect of oxygen on NOX5 expression

Results of glucose 16mM

Oxygen (%)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
2,5	7.6520	1.77255	8.0100	4.67	9.23	5
8,0	7.5360	2.94850	8.4100	2.48	9.88	5
12,0	6.1600	1.94632	6.7100	3.19	7.86	5
21,0	5.6300	1.56133	5.6200	3.51	7.38	5
In total	6.7445	2.14494	7.5200	2.48	9.88	20

Table 15 shows the Delta Cts of NOX5 in different oxygen concentrations and the glucose concentration of 16mM.

	Delta Ct NOX5
Asymptotic Significance	**.194**

Table 16 shows the result of the Kruskal-Wallis-Test, which did not show a significance (p=0.19). The group variable is oxygen (%).

Results for glucose 25mM

Oxygen (%)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
2,5	8.6025	.78978	8.6800	7.73	9.32	4
8,0	7.4280	2.50572	8.3800	2.97	8.87	5
12,0	7.2500	1.90948	8.2800	4.36	8.93	5
21,0	7.3120	2.05387	7.5000	4.37	9.82	5
In total	7.5979	1.88029	8.2800	2.97	9.82	19

Table 17 shows the Delta Cts of NOX5 in different oxygen concentrations and the glucose concentration of 25mM.

	Delta Ct NOX5
Asymptotic Significance	**.776**

Table 18 shows the result of the Kruskal-Wallis-Test, which did not show a significant variance (p=0.78). The group variable is oxygen (%).

6.3.2.2 Effect of glucose on NOX5 expression

Results for oxygen 2.5%

Glucose (mM)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
16	7.6520	1.77255	8.0100	4.67	9.23	5
25	8.6025	.78978	8.6800	7.73	9.32	4
In total	8.0744	1.43382	8.1400	4.67	9.32	9

Table 19 shows the result of the Delta Cts of NOX5 in different glucose concentrations and the oxygen concentration of 2.5%.

	Delta Ct NOX5
Asymptotic Significance (bilateral)	**.327**

Table 20 shows the result of the Mann-Whitney-U-Test, which did not show a significance (p=0.327). The group variable is glucose (mM).

Results for oxygen 8.0%

Glucose (mM)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
16	7.5360	2.94850	8.4100	2.48	9.88	5
25	7.4280	2.50572	8.3800	2.97	8.87	5
In total	7.4820	2.58023	8.3950	2.48	9.88	10

Table 21 shows the result of the Delta Cts of NOX5 in different glucose concentrations and the oxygen concentration of 8.0%.

	Delta CT NOX5
Asymptotic Significance (bilateral)	**.754**

Table 22 shows the result of the Mann-Whitney-U-Test, which did not show a significant variance (p=0.75). The group variable is glucose (mM).

Results for oxygen 12.0%

Glucose (mM)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
16	6.1600	1.94632	6.7100	3.19	7.86	5
25	7.2500	1.90948	8.2800	4.36	8.93	5
In total	6.7050	1.90634	7.2150	3.19	8.93	10

Table 23 shows the result of the Delta Cts of NOX5 in different glucose concentrations and the oxygen concentration of 12.0%.

	Delta Ct NOX5
Asymptotic Significance (bilateral)	**.251**

Table 24 shows the result of the Mann-Whitney-U-Test, which did not show a significance (p=0.25). The group variable is glucose (mM).

Results for oxygen 21.0%

Glucose (mM)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
16	5.6300	1.56133	5.6200	3.51	7.38	5
25	7.3120	2.05387	7.5000	4.37	9.82	5
In total	6.4710	1.93498	6.6600	3.51	9.82	10

Table 25 shows the result of the Delta Cts of NOX3 in different glucose concentrations and the oxygen concentration of 21.0%.

	Delta Ct NOX5
Asymptotic Significance (bilateral)	**.175**

Table 26 shows the result of the Mann-Whitney-U-Test, which did not show a significant variance (p=0.18). The group variable is glucose (mM).

6.3.3 Δ CT SOD3

Graphical overview

The expression of SOD3 under different concentrations of oxygen and glucose did not show a significant variance.

For the group of low glucose (16mM) and high glucose (25mM), there can not be shown any notable trend of the medians of the Delta Cts of SOD3 in different concentrations of oxygen.

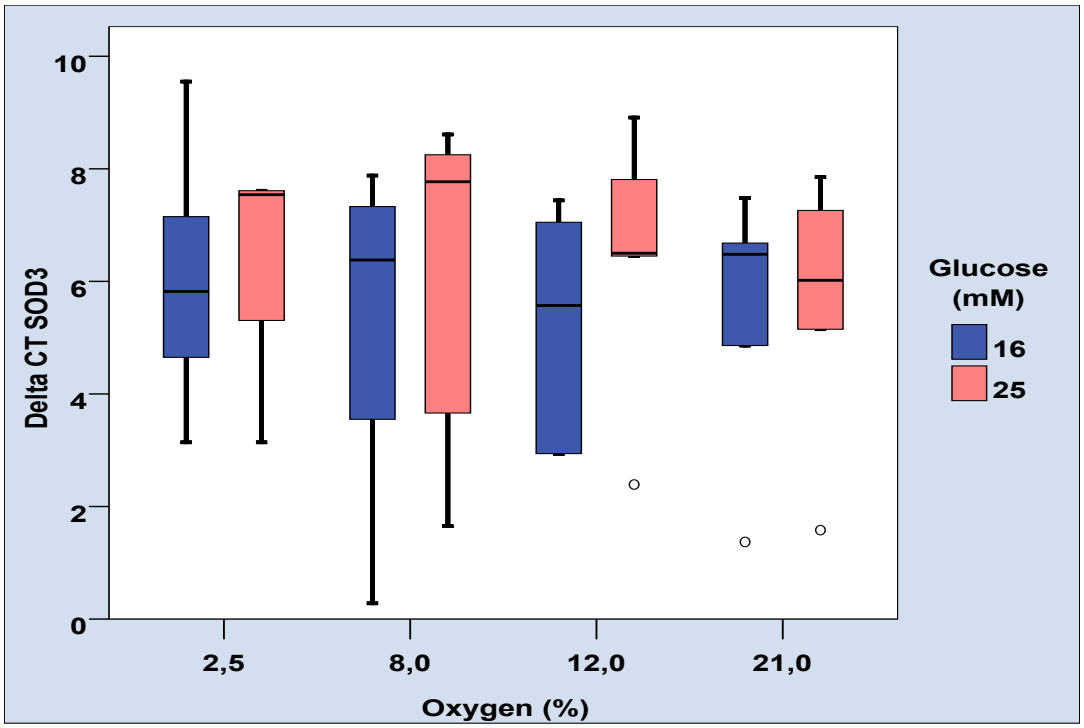

Figure 25 shows the Delta Cts of SOD3 in different concentrations of glucose and oxygen.

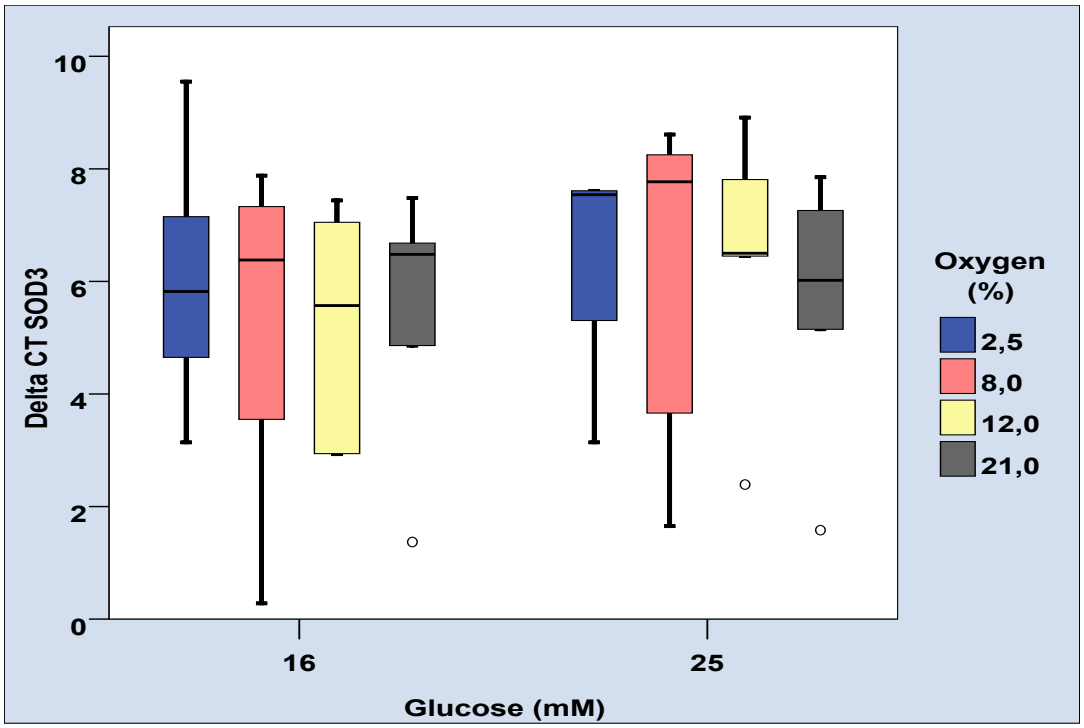

Figure 26 shows the Delta Cts of SOD3 in different concentrations of glucose and oxygen. In an 2.5%, 8.0% and 12.0% oxygen concentration, the medians of the Delta Cts of SOD3 showed lower values in 16mM glucose than in 25mM glucose, whereas in an 21.0 oxygen concentration, the medians did not show a notable difference in the different concentrations of glucose.

6.3.3.1 Effect of oxygen on SOD3 expression

Results for glucose 16mM

Oxygen (%)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
2,5	6.0620	2.44644	5.8200	3.14	9.55	5
8,0	5.0840	3.16122	6.3800	.28	7.88	5
12,0	5.1860	2.17005	5.5700	2.93	7.44	5
21,0	5.3740	2.43209	6.4800	1.37	7.48	5
In total	5.4265	2.39868	6.1000	.28	9.55	20

Table 27 shows the Delta Cts of SOD3 in different oxygen concentrations and the glucose concentration of 16mM.

	Delta Ct SOD3
Asymptotic Significance	**.962**

Table 28 shows the results of the Kruskal-Wallis-Test, which did not show a significance (p=0.96). The group variable is oxygen (%).

Results for glucose 25mM

Oxygen (%)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
2,5	6.4575	2.21265	7.5400	3.14	7.61	4
8,0	5.9880	3.13867	7.7700	1.65	8.61	5
12,0	6.4120	2.46881	6.5000	2.39	8.91	5
21,0	5.5720	2.46746	6.0200	1.58	7.85	5
In total	6.0889	2.41843	7.2600	1.58	8.91	19

Table 29 shows the Delta Cts of SOD3 in different oxygen concentrations and the glucose concentration of 25mM.

	Delta Ct SOD3
Asymptotic Significance	**.811**

Table 30 shows the results of the Kruskal-Wallis-Test, which did not show a significant variance (p=0.81). The group variable is oxygen (%).

6.3.3.2 Effect of glucose on SOD3 expression

Results for oxygen 2.5%

Glucose (mM)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
16	6.0620	2.44644	5.8200	3,14	9.55	5
25	6.4575	2.21265	7.5400	3.14	7.61	4
In total	6.2378	2.20724	7.1500	3.14	9.55	9

Table 31 shows the result of the Delta Cts of SOD3 in different glucose concentrations and the oxygen concentration of 2.5%.

	Delta Ct SOD3
Asymptotic Significance (bilateral)	**.537**

Table 32 shows the result of the Mann-Whitney-U-Test, which did not show a significance (p=0.54). The group variable is glucose (mM).

Results for oxygen 8.0%

Glucose (mM)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
16	5.0840	3.16122	6.3800	.28	7.88	5
25	5.9880	3.13867	7.7700	1.65	8.61	5
In total	5.5360	3.00779	6.8550	.28	8.61	10

Table 33 shows the result of the Delta Cts of SOD3 in different glucose concentrations and the oxygen concentration of 8.0%.

	Delta Ct SOD3
Asymptotic Significance (bilateral)	**.347**

Table 34 shows the result of the Mann-Whitney-U-Test, which did not show a significant variance (p=0.35). The group variable is glucose (mM).

Results for oxygen 12.0%

Glucose (mM)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
16	5.1860	2.17005	5.5700	2.93	7.44	5
25	6.4120	2.46881	6.5000	2.39	8.91	5
In total	5.7990	2.28459	6.4750	2.39	8.91	10

Table 35 shows the result of the Delta Cts of SOD3 in different glucose concentrations and the oxygen concentration of 12.0%.

	Delta Ct SOD3
Asymptotic Significance (bilateral)	**.465**

Table 36 shows the result of the Mann-Whitney-U-Test, which did not show a significance (p=0.47). The group variable is glucose (mM).

Results for oxygen 21.0%

Glucose (mM)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
16	5.3740	2.43209	6.4800	1.37	7.48	5
25	5.5720	2.46746	6.0200	1.58	7.85	5
In total	5.4730	2.31209	6.2500	1.37	7.85	10

Table 37 shows the result of the Delta Cts of SOD3 in different glucose concentrations and the oxygen concentration of 21.0%.

	Delta Ct SOD3
Asymptotic Significance (bilateral)	**.754**

Table 38 shows the result of the Mann-Whitney-U-Test, which did not show a significant variance (p=0.75). The group variable is glucose (mM).

6.3.4 Δ CT MMP2

Graphical overview

The expression of MMP2 under different concentrations of oxygen and glucose did not show a significant variance.

For the group of low glucose (16mM) and high glucose (25mM), there can not be shown any notable trend of the Delta Cts of MMP2 in different concentrations of oxygen.

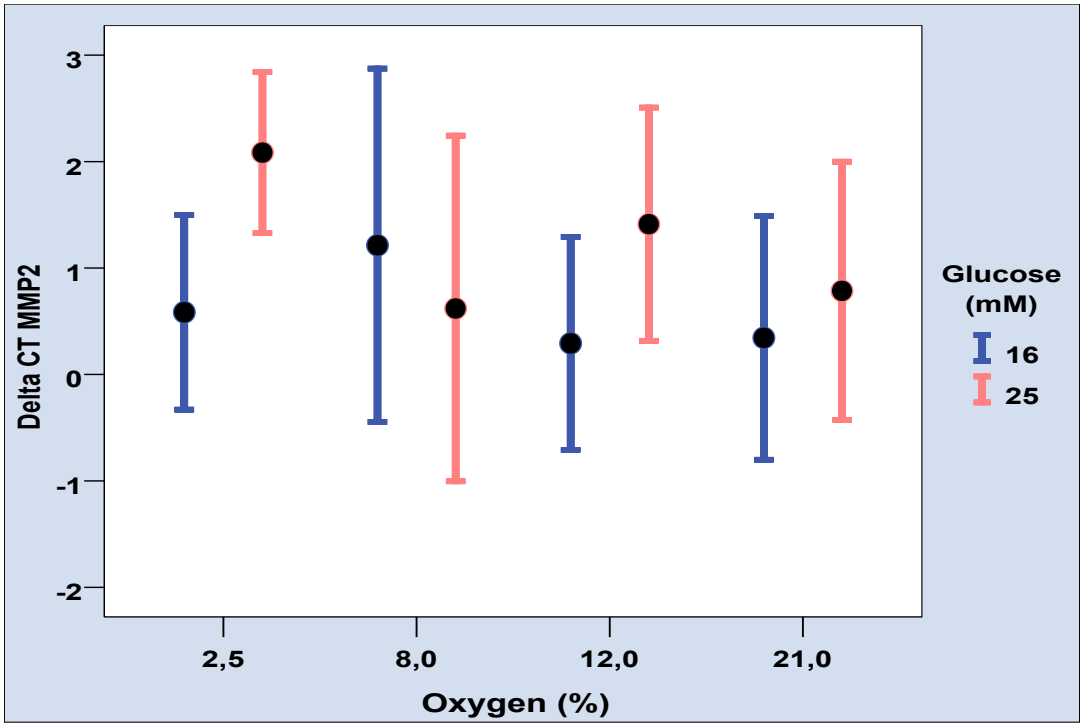

Figure 27 shows the Delta Cts of MMP2 in different concentrations of glucose and oxygen.

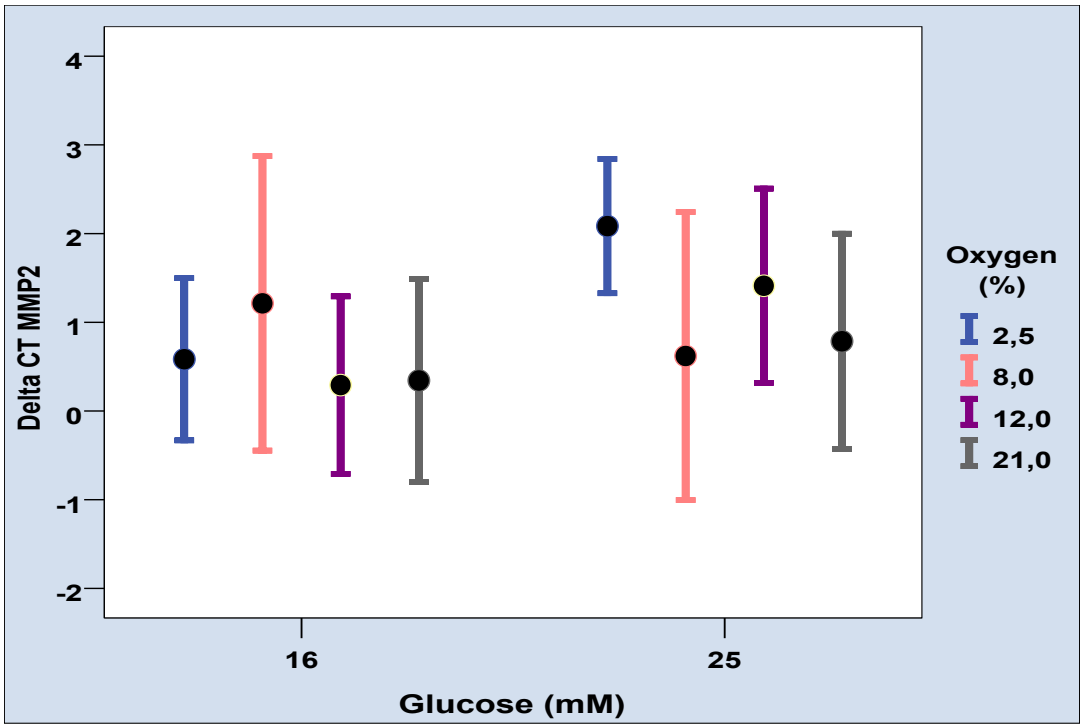

Figure 28 shows the Delta Cts of MMP2 in different concentrations of glucose and oxygen. In an 2.5%, 12.0% and 21.0% oxygen concentration, of the Delta Cts of MMP2 showed lower values in 16mM glucose than in 25mM glucose, whereas in an 8.0% oxygen concentration, the Delta Cts of MMP2 showed higher values in a low glucose concentration.

6.3.4.1 Effect of oxygen on MMP2 expression

Results for glucose 16mM

Oxygen (%)	Mean value	Standard-deviation	Standard error of mean value	Median	Minimum	Maximum	N
2,5	.5840	2.04521	.91465	.9700	-2.65	2.79	5
8,0	1.2140	3.71149	1.65983	2.1400	-4.85	4.43	5
12,0	.2920	2.23825	1.00098	-.1800	-2.28	3.37	5
21,0	.3440	2.56196	1.14574	.9800	-3.55	2.97	5
In total	.6085	2.52162	.56385	.9750	-4.85	4.43	20

Table 39 shows the Delta Cts of MMP2 in different oxygen concentrations and the glucose concentration of 16mM.

	Significance
Between the groups In total	**.946**

Table 40 shows the result of the ONEWAY ANOVA for Delta Ct of MMP2, which is not significant (p=0.95).

Results for glucose 25mM

Oxygen (%)	Mean value	Standard-deviation	Standard-error of mean value	Median	Minimum	Maximum	N
2,5	2.0850	1.51359	.75680	1.7650	.76	4.05	4
8,0	.6200	3.63079	1.62374	.5900	-4.60	5.31	5
12,0	1.4120	2.44941	1.09541	.9700	-1.99	3.90	5
21,0	.7860	2.71177	1.21274	.4700	-3.30	3.46	5
In total	1.1805	2.57019	.58964	.9700	-4.60	5.31	19

Table 41 shows the Delta Cts of MMP2 in different oxygen concentrations and the glucose concentration of 25mM.

	Significance
Between the groups In total	**.853**

Table 42 shows the result of the ONEWAY ANOVA for Delta Ct of MMP2, which did not show a significant variance (p=0.85).

6.3.4.2 Effect of glucose on MMP2 expression

Results for oxygen 2.5%

Glucose (mM)	Mean value	Standard-deviation	Standard-error of mean value	Median	Minimum	Maximum	N
16	.5840	2.04521	.91465	.9700	-2.65	2.79	5
25	2.0850	1.51359	.75680	1.7650	.76	4.05	4
In total	1.2511	1.89113	.63038	1.0400	-2.65	4.05	9

Table 43 shows the result of the Delta Cts of MMP2 in different glucose concentrations and the oxygen concentration of 2.5%. The result of the t-Test did not show a significant variance (p=0.26).

Results for oxygen 8.0%

Glucose (mM)	Mean value	Standard-deviation	Standard-error of mean value	Median	Minimum	Maximum	N
16	1.2140	3.71149	1.65983	2.1400	-4.85	4.43	5
25	.6200	3.63079	1.62374	.5900	-4.60	5.31	5
In total	.9170	3.47552	1.09906	1.3650	-4.85	5.31	10

Table 44 shows the result of the Delta Cts of MMP2 in different glucose concentrations and the oxygen concentration of 8.0%. The result of the t-Test did not show a significant variance (p=0.80).

Results for oxygen 12.0%

Glucose (mM)	Mean value	Standard-deviation	Standard-error of mean value	Median	Minimum	Maximum	N
16	.2920	2.23825	1.00098	-.1800	-2.28	3.37	5
25	1.4120	2.44941	1.09541	.9700	-1.99	3.90	5
In total	.8520	2.28943	.72398	.7300	-2.28	3.90	10

Table 45 shows the result of the Delta Cts of MMP2 in different glucose concentrations and the oxygen concentration of 12.0%. The result of the t-Test did not show a significant variance (p=0.47).

Results for oxygen 21.0%

Glucose (mM)	Mean value	Standard-deviation	Standard-error of mean value	Median	Minimum	Maximum	N
16	.3440	2.56196	1.14574	.9800	-3.55	2.97	5
25	.7860	2.71177	1.21274	.4700	-3.30	3.46	5
In total	.5650	2.49795	.78992	.7250	-3.55	3.46	10

Table 46 shows the result of the Delta Cts of MMP2 in different glucose concentrations and the oxygen concentration of 21.0%. The result of the t-Test did not show a significant variance (p=0.79).

6.3.5 Δ CT MMP7

Graphical overview

The expression of MMP7 under different concentrations of oxygen and glucose did not show a significant variance.

For the group of low glucose (16mM), there may be a decreasing trend of the medians from 2.5% to 12.0% of oxygen, but in contrary to that, in the range of 12.0% to 21.0% there may be a increasing trend of the ΔCt values with increasing oxygen concentration. This decrease of the ΔCt of the low glucose group reflects a higher expression of the gene, so MMP7 in a glucose concentration of 16mM may be higher expressed in 12.0% of oxygen.

In the group of high glucose (25mM) the medians did not show a notable trend with increasing oxygen concentrations, with the exception of remarkable low values in the group of 12% oxygen concentration, even in 15mM glucose.

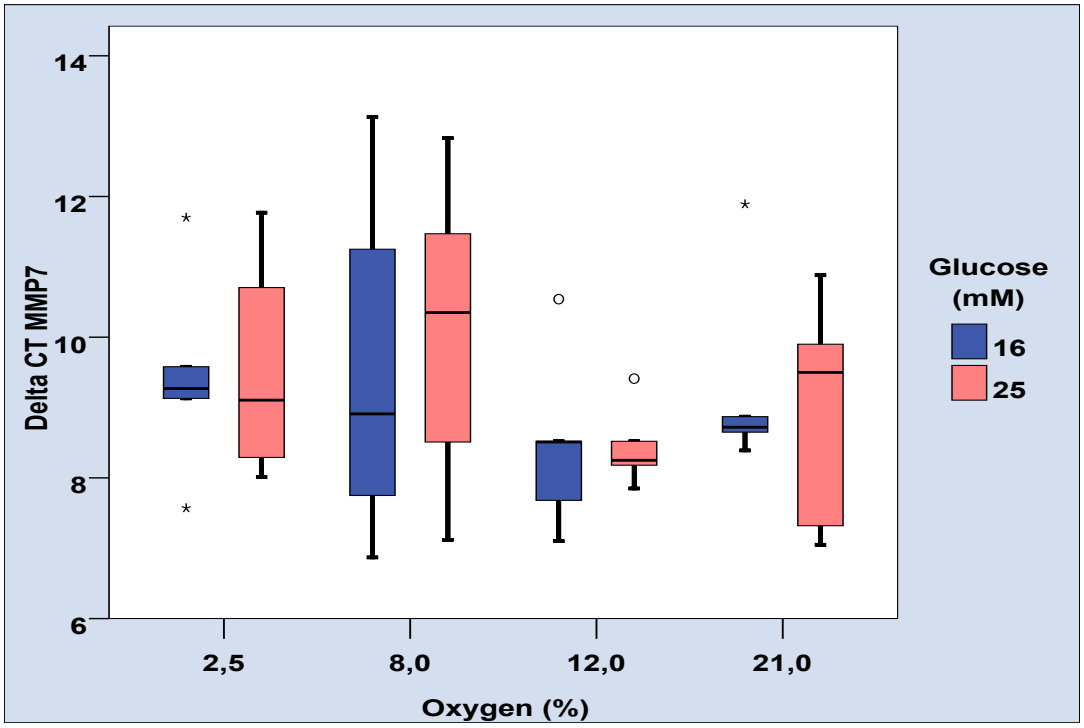

Figure 29 shows the Delta Cts of MMP7 in different concentrations of glucose and oxygen.

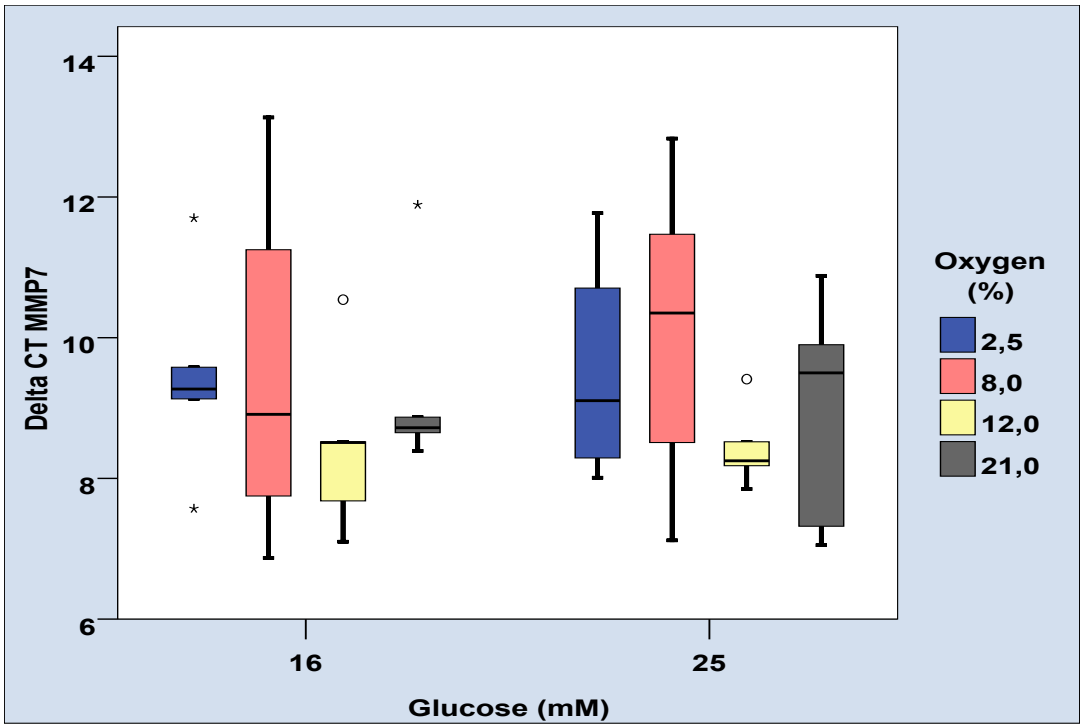

Figure 30 shows the Delta Cts of MMP7 in different concentrations of glucose and oxygen. In an 8.0% oxygen concentration, the medians of the Delta Cts of MMP7 showed lower values in 16mM glucose than in 25mM glucose, whereas in an 2.5%, 12.0% and 21% oxygen concentration, the medians did not show a notable difference under the different concentrations of glucose.

6.3.5.1 Effect of oxygen on MMP7 expression

Results for glucose 16mM

Oxygen (%)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
2,5	9.4500	1.47890	9.2700	7.57	11.70	5
8,0	9.5820	2.57552	8.9100	6.87	13.13	5
12,0	8.4700	1.30288	8.5100	7.10	10.54	5
21,0	9.3040	1.45602	8.7200	8.39	11.89	5
In total	9.2015	1.69072	8.7950	6.87	13.13	20

Table 47 shows the Delta Cts of MMP7 in different oxygen concentrations and the glucose concentration of 16mM.

	Delta Ct MMP7
Asymptotic Significance	**.557**

Table 48 shows the result of the Kruskal-Wallis-Test, which did not show a significant variance (p=0.56). The group variable is oxygen (%).

Results for glucose 25mM

Oxygen (%)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
2,5	9.4975	1.65906	9.1050	8.01	11.77	4
8,0	10.0560	2.27986	10.3500	7.12	12.83	5
12,0	8.4420	.59141	8.2500	7.85	9.41	5
21,0	8.9300	1.67293	9.5000	7.05	10.88	5
In total	9.2174	1.64809	8.5700	7.05	12.83	19

Table 49 shows the Delta Cts of MMP7 in different oxygen concentrations and the glucose concentration of 25mM.

	Delta Ct MMP7
Asymptotic Significance	**.529**

Table 50 shows the result of the Kruskal-Wallis-Test, which did not show a significance (p=0.53). The group variable is oxygen (%).

6.3.5.2 Effect of glucose on MMP7 expression

Results for oxygen 2.5%

Glucose (mM)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
16	9.4500	1.47890	9.2700	7.57	11.70	5
25	9.4975	1.65906	9.1050	8.01	11.77	4
In total	9.4711	1.45821	9.2700	7.57	11.77	9

Table 51 shows the result of the Delta Cts of MMP7 in different glucose concentrations and the oxygen concentration of 2.5%.

	Delta Ct MMP7
Asymptotic Significance (bilateral)	**.806**

Table 52 shows the result of the Mann-Whitney-U-Test, which did not show a significance (p=0.81). The group variable is glucose (mM).

Results for oxygen 8.0%

Glucose (mM)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
16	9.5820	2.57552	8.9100	6.87	13.13	5
25	10.0560	2.27986	10.3500	7.12	12.83	5
In total	9.8190	2.30666	9.6300	6.87	13.13	10

Table 53 shows the result of the Delta Cts of MMP7 in different glucose concentrations and the oxygen concentration of 8.0%.

	Delta Ct MMP7
Asymptotic Significance (bilateral)	**.754**

Table 54 shows the result of the Mann-Whitney-U-Test, which did not show a significant variance (p=0.75). The group variable is glucose (mM).

Results for oxygen 12.0%

Glucose (mM)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
16	8.4700	1.30288	8.5100	7.10	10.54	5
25	8.4420	.59141	8.2500	7.85	9.41	5
In total	8.4560	.95400	8.3800	7.10	10.54	10

Table 55 shows the result of the Delta Cts of MMP7 in different glucose concentrations and the oxygen concentration of 12.0%.

	Delta Ct MMP7
Asymptotic Significance (bilateral)	**.834**

Table 56 shows the result of the Mann-Whitney-U-Test, which did not show a significance (p=0.83). The group variable is glucose (mM).

Results for oxygen 21.0%

Glucose (mM)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
16	9.3040	1.45602	8.7200	8.39	11.89	5
25	8.9300	1.67293	9.5000	7.05	10.88	5
In total	9.1170	1.49162	8.7950	7.05	11.89	10

Table 57 shows the result of the Delta Cts of MMP7 in different glucose concentrations and the oxygen concentration of 21.0%.

	Delta Ct MMP7
Asymptotic Significance (bilateral)	**.917**

Table 58 shows the result of the Mann-Whitney-U-Test, which did not show a significant variance (p=0.92). The group variable is glucose (mM).

6.3.6 Δ CT MMP9

Graphical overview

The expression of MMP-9 under different concentrations of oxygen and glucose did not show a significant variance.

For the group of low glucose (16mM), there may be an increasing trend of the Delta Cts from 8.0% to 21.0% of oxygen, but in contrary to that, in the range of 2.5% to 8.0% there may be a decreasing trend of the ΔCt values with increasing oxygen concentration. In the group of high glucose (25mM), the medians did not show a notable trend with increasing oxygen concentrations.

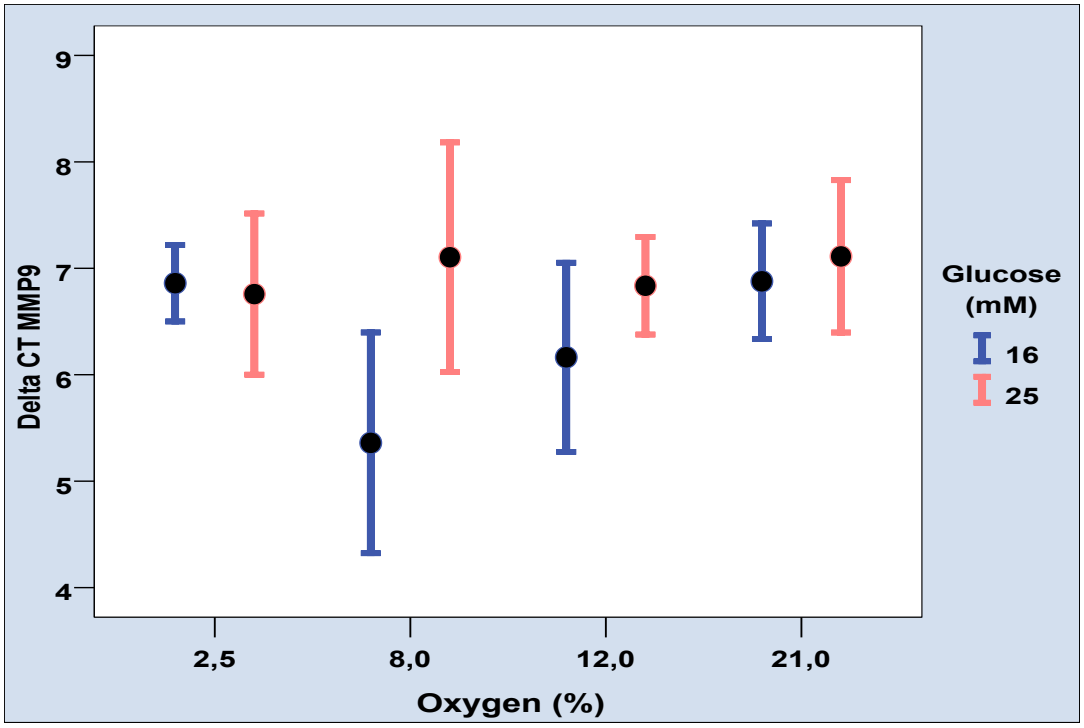

Figure 30 shows the Delta Cts of MMP9 in different concentrations of glucose and oxygen.

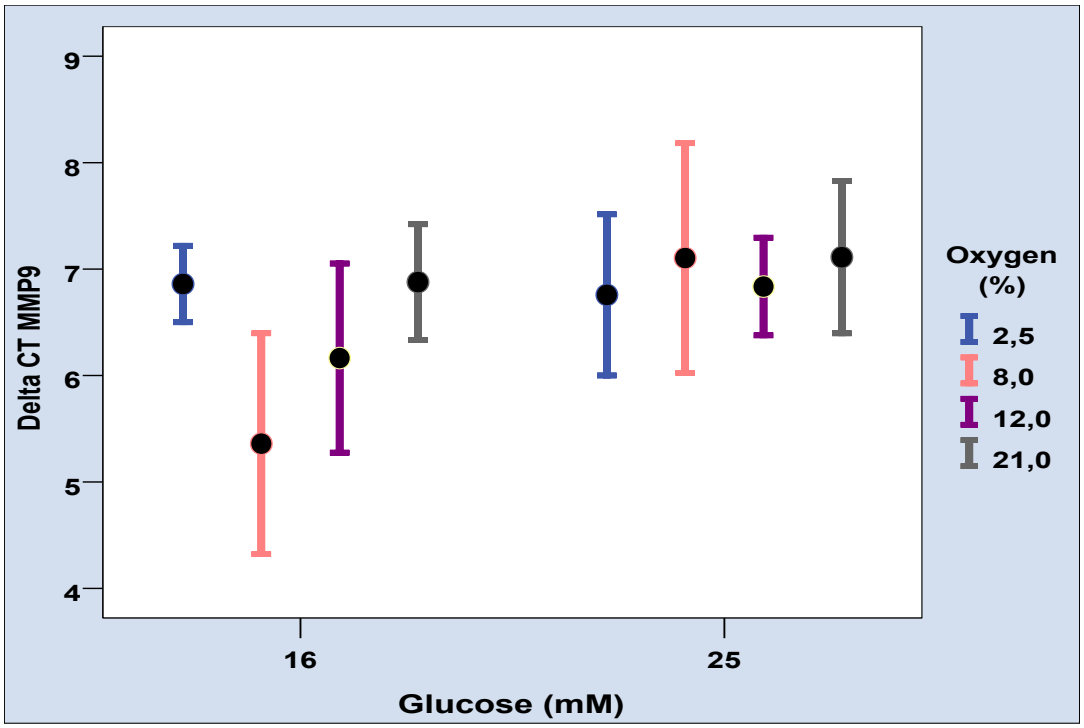

Figure 31 shows the Delta Cts of MMP9 in different concentrations of glucose and oxygen. In an 8.0% and 12.0% oxygen concentration, the Delta Cts of MMP9 showed lower values in 16mM glucose than in 25mM glucose, whereas in an 2.5 and 21% oxygen concentration, the Delta Cts did not show a notable difference under the different concentrations of glucose.

6.3.6.1 Effect of oxygen on MMP9 expression

Results for glucose 16mM

Oxygen (%)	Mean value	Standard-deviation	Standard-error of mean value	Median	Minimum	Maximum	N
2,5	6.8600	.80321	.35921	6.4700	6.11	8.14	5
8,0	5.3600	2.31952	1.03732	4.3500	3.63	9.28	5
12,0	6.1640	1.98666	.88846	6.8900	3.49	8.14	5
21,0	6.8780	1.21555	.54361	6.9600	4.97	8.17	5
In total	6.3155	1.67870	.37537	6.5700	3.49	9.28	20

Table 59 shows the Delta Cts of MMP9 in different oxygen concentrations and the glucose concentration of 16mM.

	Significance
Between the groups In total	**.462**

Table 60 shows the result of the ONEWAY ANOVA of MMP9, which did not a significance (p=0.46).

Results for glucose 25mM

Oxygen (%)	Mean value	Standard-deviation	Standard-error of mean value	Median	Minimum	Maximum	N
2,5	6.7575	1.51364	.75682	6.8700	4.81	8.48	4
8,0	7.1040	2.41264	1.07897	6.3200	4.56	10.27	5
12,0	6.8360	1.02490	.45835	7.1300	5.08	7.77	5
21,0	7.1120	1.59976	.71543	7.6500	4.99	8.60	5
In total	6.9626	1.58211	.36296	7.0800	4.56	10.27	19

Table 61 shows the Delta Cts of MMP9 in different oxygen concentrations and the glucose concentration of 25mM.

	Significance
Between the groups In total	**.984**

Table 62 shows the result of the ONEWAY ANOVA of MMP9, which did not show a significant variance (p=0.98).

6.3.6.2 Effect of glucose on MMP9 expression

Results for oxygen 2.5%

Glucose (mM)	Mean value	Standard-deviation	Standard-error of mean value	Median	Minimum	Maximum	N
16	6.8600	.80321	.35921	6.4700	6.11	8.14	5
25	6.7575	1.51364	.75682	6.8700	4.81	8.48	4
In total	6.8144	1.08842	.36281	6.6600	4.81	8.48	9

Table 63 shows the result of the Delta Cts of MMP9 in different glucose concentrations and the oxygen concentration of 2.5%. The result of the t-Test did not show a significant variance (p=0.89).

Results for oxygen 8.0%

Glucose (mM)	Mean value	Standard-deviation	Standard-error of mean value	Median	Minimum	Maximum	N
16	5.3600	2.31952	1.03732	4.3500	3.63	9.28	5
25	7.1040	2.41264	1.07897	6.3200	4.56	10.27	5
In total	6.2320	2.41311	.76309	5.5250	3.63	10.27	10

Table 64 shows the result of the Delta Cts of MMP9 in different glucose concentrations and the oxygen concentration of 8.0%. The result of the t-Test did not show a significant variance (p=0.28).

Results for oxygen 12.0%

Glucose (mM)	Mean value	Standard-deviation	Standard-error of mean value	Median	Minimum	Maximum	N
16	6.1640	1.98666	.88846	6.8900	3.49	8.14	5
25	6.8360	1.02490	.45835	7.1300	5.08	7.77	5
In total	6.5000	1.53181	.48440	7.0650	3.49	8.14	10

Table 65 shows the result of the Delta Cts of MMP9 in different glucose concentrations and the oxygen concentration of 12.0%. The result of the t-Test did not show a significant variance (p=0.52).

Results for oxygen 21.0%

Glucose (mM)	Mean value	Standard-deviation	Standard-error of mean value	Median	Minimum	Maximum	N
16	6.8780	1.21555	.54361	6.9600	4.97	8.17	5
25	7.1120	1.59976	.71543	7.6500	4.99	8.60	5
In total	6.9950	1.34512	.42536	7.2900	4.97	8.60	10

Table 66 shows the result of the Delta Cts of MMP9 in different glucose concentrations and the oxygen concentration of 21.0%. The result of the t-Test did not show a significant variance (p=0.80).

6.3.7 Δ CT MMP19

Graphical overview

The expression of MMP19 under different concentrations of oxygen and glucose did not show a significant variance.

For the group of low glucose (16mM), there may be a decreasing trend of the medians from 2.5% to 12.0% of oxygen, but in contrary to that, in the range of 12.0% to 21.0% there may be an increasing trend of the medians of the ΔCt values with increasing oxygen concentration. This decrease of the ΔCt of the low glucose group reflects a higher expression of the gene, so MMP19 in a glucose concentration of 16mM may be higher expressed under 12.0% of oxygen.

In the group of high glucose (25mM) the medians did not show a notable trend with increasing oxygen concentrations.

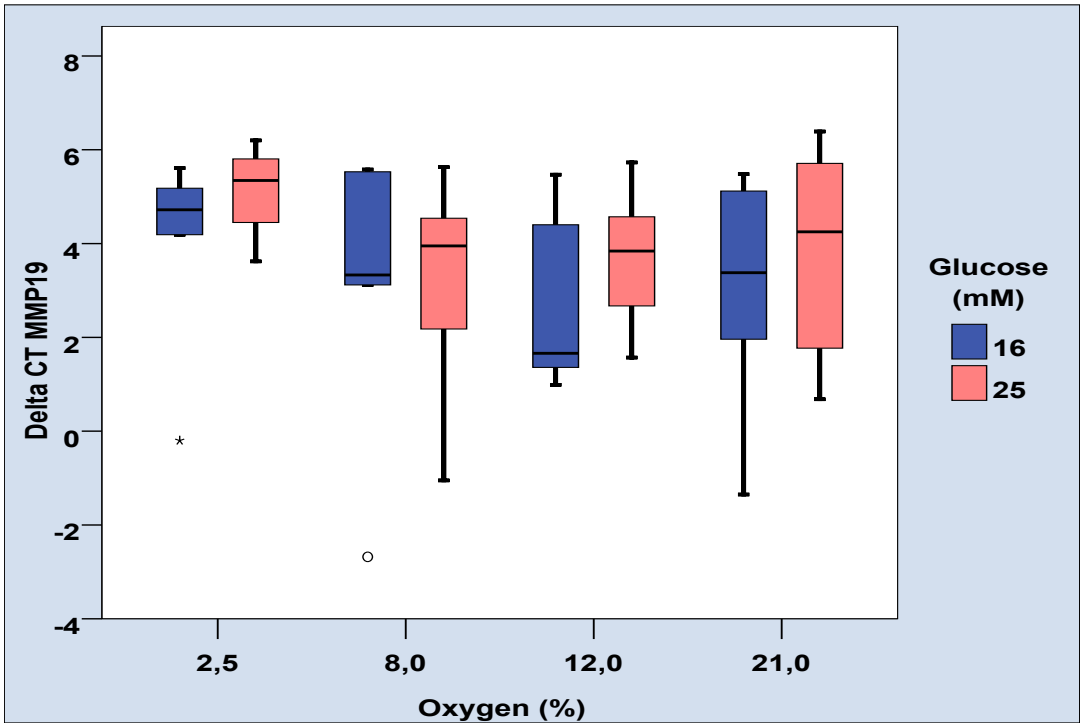

Figure 32 shows the Delta Cts of MMP19 in different concentrations of glucose and oxygen.

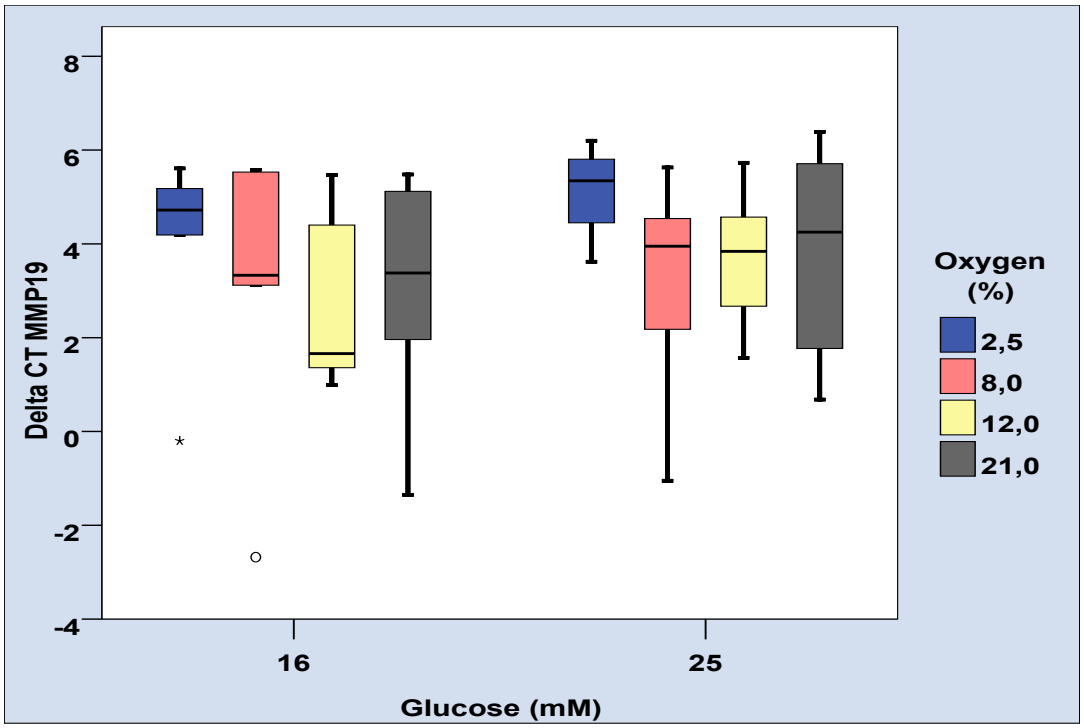

Figure 33 shows the Delta Cts of MMP19 in different concentrations of glucose and oxygen. In every different glucose concentration the medians of the Delta Cts of MMP19 showed lower values in 16mM glucose than in 25mM glucose, eventhough those variations are partially very slightly and are not significant.

6.3.7.1 Effect of oxygen on MMP19 expression

Results for glucose 16mM

Oxygen (%)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
2,5	3.9000	2.35207	4.7200	-.20	5.61	5
8,0	2.9760	3.37046	3.3300	-2.68	5.58	5
12,0	2.7760	2.02085	1.6600	.99	5.47	5
21,0	2.9180	2.77307	3.3800	-1.35	5.48	5
In total	3.1425	2.49837	3.7850	-2.68	5.61	20

Table 67 shows the Delta Cts of MMP19 in different oxygen concentrations and the glucose concentration of 16mM.

	Delta Ct MMP19
Asymptotic Significance	**.776**

Table 68 shows the result of the Kruskal-Wallis-Test, which did not show a sgnificance (p=0.78). The group variable is oxygen (%).

Results for glucose 25mM

Oxygen (%)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
2,5	5.1275	1.08411	5.3450	3.62	6.20	4
8,0	3.0500	2.61024	3.9500	-1.05	5.63	5
12,0	3.6760	1.61959	3.8400	1.57	5.73	5
21,0	3.7600	2.47012	4.2500	.68	6.39	5
In total	3.8389	2.04932	4.2500	-1.05	6.39	19

Table 69 shows the Delta Cts of MMP19 in different oxygen concentrations and the glucose concentration of 25mM.

	Delta Ct MMP19
Asymptotic Significance	**.625**

Table 70 shows the result of the Kruskal-Wallis-Test, which did not show a significant variance (p=0.63). The group variable is oxygen (%).

6.3.7.2 Effect of glucose on MMP19 expression

Results for oxygen 2.5%

Glucose (mM)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
16	3.9000	2.35207	4.7200	-.20	5.61	5
25	5.1275	1.08411	5.3450	3.62	6.20	4
In total	4.4456	1.90405	5.1800	-.20	6.20	9

Table 71 shows the result of the Delta Cts of MMP19 in different glucose concentrations and the oxygen concentration of 2.5%.

	Delta Ct MMP19
Asymptotic Significance (bilateral)	**.327**

Table 72 shows the result of the Mann-Whitney-U-Test, which did not show a significance (p=0.33). The group variable is glucose (mM).

Results for oxygen 8.0%

Glucose (mM)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
16	2.9760	3.37046	3.3300	-2.68	5.58	5
25	3.0500	2.61024	3.9500	-1.05	5.63	5
In total	3.0130	2.84228	3.6400	-2.68	5.63	10

Table 73 shows the result of the Delta Cts of MMP19 in different glucose concentrations and the oxygen concentration of 8.0%.

	Delta Ct MMP19
Asymptotic Significance (bilateral)	**.917**

Table 74 shows the result of the Mann-Whitney-U-Test, which did not show a significant variance (p=0.92). The group variable is glucose (mM).

Results for oxygen 12.0%

Glucose (mM)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
16	2.7760	2.02085	1.6600	.99	5.47	5
25	3.6760	1.61959	3.8400	1.57	5.73	5
In total	3.2260	1.79049	3.2550	.99	5.73	10

Table 75 shows the result of the Delta Cts of MMP19 in different glucose concentrations and the oxygen concentration of 12.0%.

	Delta Ct MMP19
Asymptotic Significance (bilateral)	**.347**

Table 76 shows the result of the Mann-Whitney-U-Test, which did not show a significance (p=0.35). The group variable is glucose (mM).

Results for oxygen 21.0%

Glucose (mM)	Mean value	Standard-deviation	Median	Minimum	Maximum	N
16	2.9180	2.77307	3.3800	-1.35	5.48	5
25	3.7600	2.47012	4.2500	.68	6.39	5
In total	3.3390	2.51524	3.8150	-1.35	6.39	10

Table 77 shows the result of the Delta Cts of MMP19 in different glucose concentrations and the oxygen concentration of 21.0%.

	Delta Ct MMP19
Asymptotic Significance (bilateral)	**.602**

Table 78 shows the result of the Mann-Whitney-U-Test, which could not show a significant variance (p=0.60). The group variable is glucose (mM).

7 Diskussion

The role of the placental enzymes under diabetic conditions as there is hyperglycemia and hypoxia, is not yet sufficiently investigated. Our aim was to find out wheather the MMPs and the enzymatic antioxidants undergo a deregulation of their gene-expression under diabetic conditions.
It is important to remark the fact that RT-PCR results do only reflect the gene-expression and can not make a point about the *in vivo* processes.

7.1 MMPs

An altered expression of the MMPs as markers for tissue remodeling during invasion in the placenta caused by increased blood sugar levels and hypoxia, leads to changes in proliferation, differentiation and invasion of the trophoblast cells, because these cells are depending on the enzymatic lysis of the extracellular matrix by the MMPs. This leads to a deficient anchoring and implantation of the placenta and as a consequence of that the risk of missed abortions will increase.
Eventhough the examined parameters did not show a significant result, we suggest that these enzymes are only a part of the mechanisms, which regulate the invasion of the trophoblast. There exist also other features that limit or promote trophoblast invasiveness, including decidual activities, growth factors, immunological mechanisms, the oxygen partial pressure and mechanisms induced by the decidua. So another aspect is that the uterine tissue may also be involved in the way that it may be modified by diabetes. As a consequence of that there may be changed conditions for the trophoblast cells to invade the uterus.
It is assumed, that the expression of MMPs itself may not be a direct indicator for invasiveness, because they may be required for the turnover of self-secreted extracellular matrix. Nonetheless an alteration of the MMPs may be regulated at the “protein-level”, independent of the RNA-level, due to the fact that the MMPs first have to get activated by cleavage. As a consequence of that the RNA may not show significant differences, but there may still be alterations of the enzymatic activity at the protein-level.
For the fetus and the pregnancy an altered and insufficient trophoblast invasion may cause a deficient fetal supply and an increased risk of pregnancy disorders.

7.2 Enzyamatic antioxidants

As a consequence of high metabolic activity in the feto-placental compartment, pregnancy is a state of oxidative stress, because oxidants have many physiological functions like the control of cellular fate and cellular signaling. But in normal pregnancies a parallel increase in antioxidative activity balances the elevated ROS levels. Low oxygen environment like in early pregnancy is assumed to limit ROS and this may prevent the placenta from damages caused by these oxidative stress. This is an important fact, because the placenta as a tissue of high metabolic activity and cell division is very sensitive. In the late first trimester of pregnancy an increase in antioxidant capacity and antioxidant enzyme activity can be determined.

In GDM a systemic oxidative stress caused by elevated glucose levels, can lead to cellular damage by acting on DNA, lipids and proteins, as well as lower trophoblast growth and proliferation in the first trimester. Even the MMPs will get influenced by this increased oxidative stress and as a consequence the invasion of the trophoblast cells will get altered, too.

The remodeling of uteroplacental arteries and the invasion of the trophoblast cells are processes associated to the mature placental function that can be highly affected by hyperglycemia-induced nitrative and oxidative stress.

The consequences for the fetus may be disorders in the development caused by increased ROS levels. The fetus may have to make an effort in compensating this increased level of oxidative stress. The regular remodeling of the uteroplacental arteries may also be affected by the changes in the invading trophoblast amount as well as the placental and embryonic growth may get reduced.

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9 Figure Legends

Figure 1: http://www.rrc.com/about/ivf-lab.php, last visit: 21.07.2012

Figure 2: http://www.biosci.uga.edu/almanac/bio_103/notes/apr_11.html., last visit: 22.07.2012

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Figure 5: Kay H, Nelson DM, Wang Y. The Placenta: From Development to Disease. 1. Aufl. John Wiley & Sons; 2011, chapter 30, page 234, table 30.3

Figure 6: Kay H, Nelson DM, Wang Y. The Placenta: From Development to Disease. 1. Aufl. John Wiley & Sons; 2011, chapter 30, page 233, table 30.2

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Figure 19: http://www.qiagen.com/literature/render.aspx?id=23490, last visit: 26.07.2012

10 Abbreviations

Tri: Tri-Reagent (DNA/RNA/Protein Isolation Reagent), Molecular Research Center (MRC), #TR 118, contain phenol and guanidine thiocyanate

BCP: Phase Separation Reagent, 1-Bromo-3-Chloropropane, MRC, #BP-151

RT-PCR: real time or also known as quantitative reverse transcription polymerase chain reaction

Aqua-dest: distilled water without imurities, trace elements or ions

mRNA: messanger RNA

TNFα: tumor necrosis factor- alpha

BCP: bromchlorpropan

PDM: pre-existing diabetes mellitus

GDM: gestational diabetes mellitus

%: percent

°C: degree Celsius

µg: microgram

µl: microliter

Aqua dest.: Aqua destillata

cDNA: copy desoxyribonucleic acid

CO_2: carbon dioxide

NO: nitrogen monoxide

RNA: ribonucleic acid

T1D: diabetes mellitus type 1

UV: ultraviolet

mM: molar Mass

g: gram

ROS: reactive oxygen species

NOX5: nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 5

SOD3: superoxide dismutase 3

GPX3: gluthatione peroxidase 3

M-MLV RT: RNA-dependent DNA polymerase

VEGF: vascular endothelial growth factor

FGF: fibroblast growth factor

PIGF: placental growth factor

MMP: matrix metalloproteinase

ECM: extracellular matrix

pO_2: oxygen partial pressure

ml: milliliter

TNFα: tumor necrosis factor alpha

DNA: desoxyribonucleic acid

RNA: ribonucleic acid

HCG: human chorionic gonadotropin

HCT: human chorionic thyrotropin

HPL: human placental lactogen

IgG: immunoglobulin G

DMEM: Dulbecco's modified Eagle Medium

Ct: cycle threshold

mmHg: millimeter of mercury

m^2: square meter

DUOX 1: dual oxidase 1

ET-1: Endothelial cells

Rac-1: Ras-related C3 botulinum toxin substrate

Δ : Delta

CuZn SOD: Cu,Zn-superoxide dismutase

CAT: catalization

H_2O_2: hydrogen peroxide

H_2O: water

O_2: oxygen