Diplomarbeit

Influence of Insulin on ER-stress in Human First Trimester Trophoblast
An *in vitro* study

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Graz, am 16.02.2021 *Sanela Nikodijevic eh.*

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LIST OF ABBREVIATIONS

ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
BFA	brefeldin A
CHOP	C/EBP homologous protein
Ct	cycle treshhold
eIF2α	eukaryotic translation initiation factor 2
ER	endoplasmatic reticulum
ERAD	ER-associated degradation
EVT	extravillous cytotrophoblast
FBS	fetal bovine serum
GDM	gestational diabetes mellitus
GRP78	78-kDa glucose regulated protein
HBSS	Hank's Balanced Salt Solution
hCG	human chorionic gonadotropin
HGPRT	hypoxanthine-guanine-phosphoribosyl- transferase
HIFs	hypoxia inducible factors
IRE1	inositol requiring protein 1
mTOR	mammalian target of rapamycin
ns	not significant
PERK	protein kinase R-like endoplasmatic reticulum kinase
PKR	protein kinase RNA
PPIA	peptidyl-prolyl-isomerase A
RT-qPCR	quantitative real-time polymerase chain reaction
SCT	syncytiotrophoblast
sXBP1	spliced X-box binding protein 1
TBE	1x Tris Buffer EDTA
TBP	TATA-box binding protein
TM	tunicamycin
UPR	unfolded protein response
VCT	villous cytotrophoblast
XBP1	X-box binding protein 1

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ZUSAMMENFASSUNG

Die Plazenta bildet die Verbindung zwischen Mutter und dem heranwachsenden Kind. In der Schwangerschaft ändert sich nachweislich der Stoffwechsel der Mutter, unter anderem der Glukosestoffwechsel durch eine erhöhte Insulinresistenz. Diese Umstände nehmen schon am Beginn der Schwangerschaft Einfluss auf die Plazenta und damit auf mögliche Schwangerschaftskomplikationen wie Gestationsdiabetes und Präeklampsie. Diese Erkrankungen haben nicht nur unmittelbare Konsequenzen für das Ungeborene, sondern erhöhen auch das Risiko für weitere Erkrankungen wie Diabetes Mellitus und kardiovaskuläre Erkrankungen für Mutter und Kind. Eine wichtige Rolle in der zugrundeliegenden Pathophysiologie spielt ER-Stress, der durch intrazellulär angesammelte, teils fehlgefaltete Proteine ausgelöst wird. Um die intrazelluläre Homöostase wiederherzustellen antwortet die Zelle mit der Unfolded Protein Response (UPR). Diese wird durch die ER-Stress Sensoren IRE, ATF6 und PERK reguliert. Sollte die Herstellung der Homöostase nicht möglich sein, geht die Zelle in Apoptose über.

Vorherige laborinterne Versuche haben gezeigt, dass eine hohe Insulinkonzentration zu einer verminderten Phosphorylierung von eIF2α geführt hat und weißt damit auf einen stressreduzierenden Effekt von Insulin in diesem Signalweg hin. Ziel ist es daher zu untersuchen, ob Insulin einen möglichen ER-Stress reduzierenden Einfluss auf Erst- trimester Trophoblast Zellen hat, die mit ER-Stress auslösenden Faktoren inkubiert werden.

Dazu wurde ein Zellkulturmodel für Erst- trimester Trophoblast Zellen, die ACH-3P Zelllinie verwendet. Dabei handelt es sich um eine fusionierte Zelllinie aus extravillösen und intravillösen männlichen Trophoblast Zellen und der Chorionkarzinom Zelllinie AC1-1. Es wurden vier Passagen der ACH-3P Zelllinie unter physiologischen Bedingungen von 6,5% Sauerstoffkonzentration und 37 Grad Celsius kultiviert und in einem Medium mit einer pathologischen Insulinkonzentration und den Er-Stress auslösenden Faktoren Tunicamycin und Brefeldin A inkubiert. Nach 24 Stunden wurde der Einfluss des Insulins gemessen indem spezifische Marker der Signalwege in der UPR analysiert wurden. Die Signalwege wurden untersucht indem phosphoryliertem und nicht-phosphoryliertem eIF2α und IRE1α Protein mittels Westernblot gemessen wurden. Mittels RT-qPCR wurden die Expression des pro-apoptotischen Transkriptionsfaktors *CHOP*, sowie gespleißtem und ungespleißtem *XBP1* bestimmt.

Die statistische Auswertung konnte weder mittels Immunoblot, noch mit RT-qPCR eine ER-Stress Induktion durch Insulin in ACH-3P Zellen zeigen. Eine Stressinduktion der Zellen durch Behandlung der Zellen mit Brefeldin A und Tunicamycin konnte gezeigt werden. Der ER-stress Marker GRP78 war erhöht (8.6-fach und 4.7-fach, $p \leq 0.001$). Ebenfalls erhöht war das Protein IRE1α (2.2-fach und 1.9-fach, $p \leq 0.01$), die relative Expression von *CHOP* (4.8-fach und 4.9-fach, $p \leq 0.001$), *XBP1* (1.7-fach und 1.6-fach, $p \leq 0.0001$ und $p \leq 0.05$) sowie dessen Splicing (8.5-fach und 12-fach, $p \leq 0.001$).

Der Vergleich der Ergebnisse der mit ER-Stress Induktoren und Insulin behandelten Zellen zeigte jedoch keinen Unterschied im Vergleich mit den Zellen, die nur mit ER-Stress induzierenden Faktoren aber ohne Insulin inkubiert wurden. Daher kann davon ausgegangen werden, dass Insulin keinen Effekt auf ER-Stress in der Erst-trimester Trophoblast Zelllinie ACH-3P hat.

ABSTRACT

The placenta is the connection between the mother and the growing fetus. During pregnancy maternal metabolism changes, for example glucose metabolism, which translates into increased insulin resistance. These circumstances have an impact on the placenta and therefore on adverse pregnancy outcomes like gestational diabetes mellitus and pre-eclampsia. Furthermore, these diseases have not only acute consequences for the unborn but also increase the risks for cardiovascular diseases for mother and child. ER-stress plays an important role in pathophysiology of pregnancy and is induced by unfolded and misfolded proteins accumulated in the cell. To restore intracellular homeostasis, the cell responds with the so-called unfolded protein response (UPR). ER-stress sensors IRE, ATF6 and PERK regulate UPR. When homeostasis cannot be restored, apoptosis will be activated.

Preliminary results have shown that pathophysiological insulin concentration decreases eIF2α phosphorylation suggesting that insulin might reduce ER-stress. The objective of this thesis is to investigate if insulin has an ER-stress reducing effect on first trimester trophoblast cells, which have been cultured with ER-stress inducing factors.

The ACH-3P cell line was used as a model for first trimester trophoblast. It is a cell line of fused intravillous male trophoblast cells and the choriocarcinoma cell line AC1-1. Four passages of ACH-3P cell line were cultivated under physiological conditions of 6.5% O_2 and 37°C in a medium containing pathological insulin of 10nM concentration and ER-stress inducers brefeldin A and tunicamycin. After 24 hours, the effect of insulin was determined by measuring specific ER-stress markers of different signaling pathway of UPR. GRP78, phospho- and total eIF2α as well as phospho- and total IRE1α protein levels were evaluated by Western blotting. Using RT-qPCR, mRNA levels of pro-apoptotic transcription factor *CHOP* as well as spliced and non-spliced *XBP1* were measured.

Statistical analysis of immunoblot and RT-qPCR could not show an effect of insulin on ACH-3P cells. ER-stress induction with tunicamycin (TM) and brefeldin A (BFA) could be shown. ER-stress marker GRP78 was increased (8.6-fold and 4.7-fold, respectively; $p \leq 0.001$) as well as total IRE1α (2.2-fold and 1.9-fold respectively; $p \leq 0.01$) and relative expression of *CHOP* (4.8-fold and 4.9-fold, respectively, $p \leq 0.001$), *XBP1*(1.7-fold and 1.6-fold, respectively; $p \leq 0.0001$ and $p \leq 0.05$*)* and its splicing (8.5-fold and 12-fold, respectively $p \leq 0.001$).

Comparing the statistical results of ACH-3P cells incubated only with ER-stress inducers to cells incubated with ER-stress inducers TM and BFA with additional insulin, no significant difference was found. Under these circumstances, it can be considered that insulin has no effect on ER-stress in first trimester trophoblast cell line ACH-3P.

1 Introduction

1.1 The human Placenta and its Development

The mature placenta has a discoid shape with an average diameter of 22cm and 500g weight. It is a transient organ between the circulation of mother and child not only supplying the fetus with oxygen and nutrients but also acting as lungs, liver, gut, kidney and endocrine organs for the unborn. To achieve this, the placenta possesses the ability to adapt and build a large surface area for circulation and remodels the maternal uterine arteries for an optimal perfusion. The basal plate is facing the maternal endometrium while the chorion plate is facing the fetus, which is attached to it by the umbilical cord. Between the two plates is the intervillous space, a cavity filled with maternal blood. Attached to the stem villous, 30-40 villous trees are projected into the intravillous space forming lobules. Each lobule is an independent maternal-fetal exchange unit as it is located over a maternal spiral artery through the basal plate (*Figure 1*). The terminal villi of the villous trees extend the surface to 12-14m^2 (Burton and Fowden, 2015).

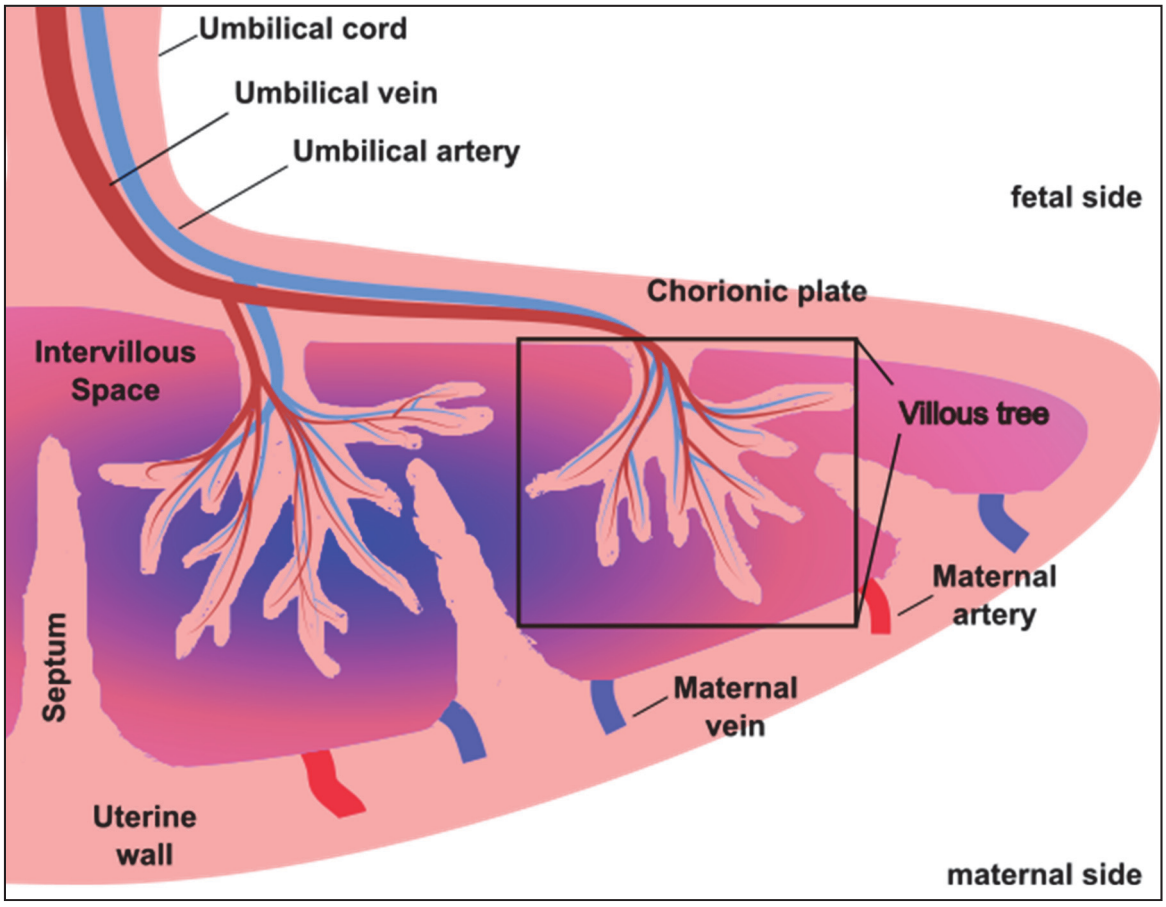

Figure 1: Organization of the mature human placenta. Modified from Slator et al. 2018.

There are two principle tissue sources for the development, the trophectoderm and the extraembryonic mesoderm. The trophectoderm forms the wall of the blastocyst and differentiates into trophoblast cells, which are the defining cells of the placenta. The extraembryonic mesoderm builds the stromal core of the placenta and is the origin for fibroblasts and the vascular network (*Figure 2*) (Burton and Fowden, 2015).

The major functions of the placenta are performed by trophoblast cells. Since their discovery, many subtypes of human trophoblast subtypes have been identified including syncytiotrophoblast (SCT), the villous cytotrophoblast (VCT) and subtypes of the extravillous cytotrophoblast (EVT).

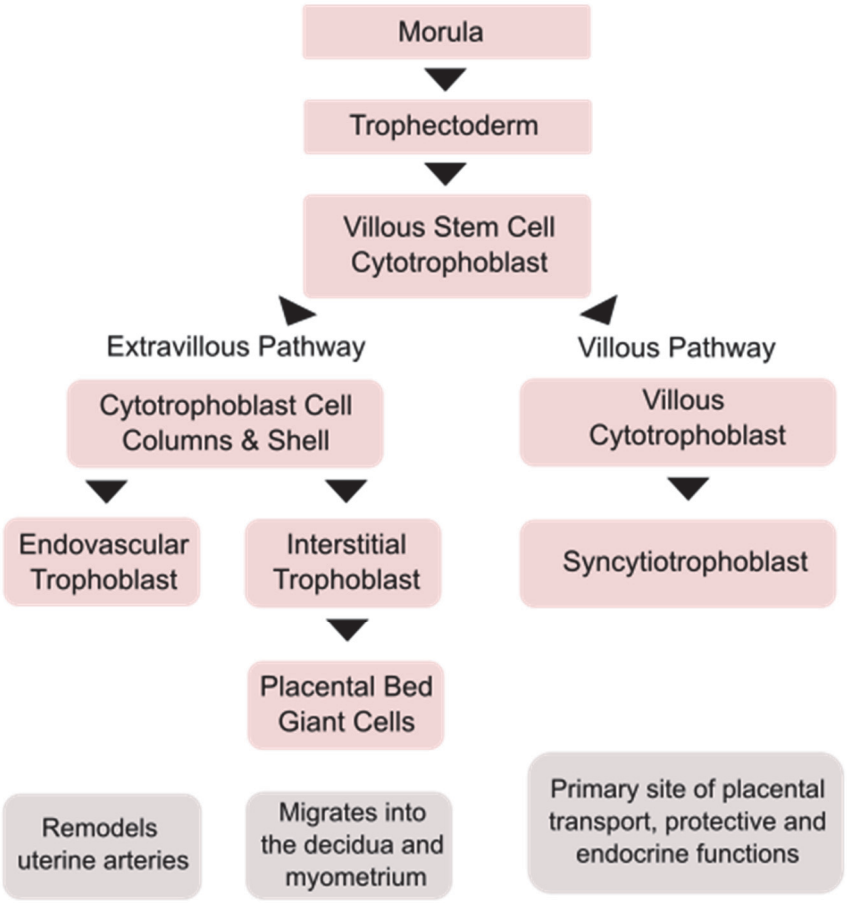

Figure 2: Differentiation of trophoblasts and their functions. Modified from Gude et al., 2004.

SCT build the epithelial cover of the villous trees and are involved in synthesis and secretion of steroids and peptide hormones. As SCT present no cellular clefts to the intravillous space, they act as protection against xenobiotics and prevent vertical transmission of pathogens. VCT lies beneath the SCT, are mitotic and express proliferative markers. EVT cells migrate into the decidua and differentiate into

interstitial EVT, migrating towards maternal spiral arteries and endovascular EVT, moving down the inside of spiral arteries (Turco and Moffett, 2019). The development and different structures found in the placenta reveals the complexity of this important organ.

1.2 The Placenta in Early Pregnancy

Early pregnancy can be viewed as an anabolic state characterized by increased food intake and energy storing whereas later on, pregnancy resembles a catabolic state, mobilizing reserves and preparing for lactation (Lain and Catalano, 2007).

Surprisingly, development of early placenta and embryo take place under low oxygen tension. Intervillous oxygen tension was reported to be about 2-3% between 8^{th} and 10^{th} week but rises steeply to >6% O_2 after 12^{th} week of gestation. This is due to physiological changes, the remodeling in maternal arteries and placental vascularization. All these processes increase O_2 tension at the end of first trimester, as uterine spiral arterioles are previously occluded by extravillous trophoblast (Chang, Wakeland and Parast, 2018). This is crucial for the placenta, since low oxygen environment is implicated as the key factor in placental development. Low oxygen tension is physiological in this stage of gestation but force the cells to adjust to this hypoxia (Patel *et al.*, 2010). There are several oxygen-sensitive signaling pathways with different downstream targets and cascades, including hypoxia inducible factors (HIFs), mammalian target of rapamycin (mTOR) and the unfolded protein response (UPR) pathway. These pathways are known to be involved in placental development and disease as they play an important role in angiogenesis, apoptosis, cell survival, proliferation and metabolism (Chang, Wakeland and Parast, 2018).

The main source for energy for placental and fetal metabolism is the carbohydrate glucose. Its transport to the fetus is regulated by placental hormones, which increase the maternal glucose concentration, build a concentration gradient and regulate the density of transporters in syncytiotrophoblast (Burton and Fowden, 2015). The human placenta expresses seven isoforms of glucose transporter, GLUT 1, 3, 4, 8, 9, 10 and 12. In early gestation, GLUT3 transporters with their high affinity to glucose play an important role for ensuring placental uptake while later in gestation GLUT1 transporter has to provide the high-volume supply for the growing fetus (Nicholas P. Illsley, 2018).

In early pregnancy, trophoblast cells produce human chorionic gonadotropin (hCG). It is secreted into the maternal circulation with peak levels about 8 weeks, followed by decreased levels from about 12th week and a rise again later in pregnancy. HCG stimulates cytotrophoblastic cell fusion as well as functional differentiation of villous trophoblasts (Gude *et al.*, 2004).

1.3 ER-Stress

The Endoplasmic Reticulum (ER) is a large, dynamic cell organelle. With a continuous membrane system including the nuclear envelope. It is therefore an organelle with many crucial functions such as synthesis and folding of transmembrane and secreted proteins. Furthermore, it is capable of lipid and steroid synthesis, carbohydrate metabolism and acts as calcium storage with a concentration of ~100–800µM compared to a typical cytosolic concentration of ~100nM. Protein translation of secretory and integral membrane proteins starts in the cytosol. The processed protein contains a signal sequence that is recognized via signal recognition particle and is recruited to the cytosolic ER where the mRNA-ribosome complex docks and the translation continues. Through the translocon, a channel which spans the lipid bilayer, the protein enters the ER lumen. At this point, if the protein is destined to be an integral protein, the translation is paused and the protein will be shifted and anchored within the phospholipid bilayer. If the protein is destined to be secreted, it has to undergo folding and modification in the ER lumen by chaperons and folding enzymes (Schwarz and Blower, 2016).

The conditions, which impose stress on cells such as hypoxia, starvation or infection challenges the folding capacity of the cell by the disturbance of many homeostatic processes. Hindering protein folding can lead to misfolded, unfolded or aggregated proteins. The cell responds to this ER-stress with the activation of sensors leading to signaling cascades to restore homeostasis. To cope with stress, UPR is activated to synthesize new proteins and reduce the general protein synthesis (Iurlaro and Muñoz-Pinedo, 2016). UPR also includes a process called ER-associated degradation (ERAD) by which protein degradation through autophagy and enhanced clearance of unfolded proteins is triggered to restore homeostasis within the cell (Iurlaro and Muñoz-Pinedo, 2016). Unfolded or misfolded proteins in the ER lumen are recognized and delivered to the cytosol for degradation by the ubiquitin dependent proteasome system (Yamamoto *et al.*,

2007). UPR plays an important role in metabolism and development, chronic ER-stress and inflammation, immunology and aging as the UPR becomes dysfunctional and causes tissue failure (Frakes and Dillin, 2017).

Three main transmembrane ER- sensors regulate UPR: inositol requiring protein 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase RNA (PKR)-like ER kinase (PERK) (*Figure 3).* ER sensors therefore have a luminal domain for sensing protein- folding environment in ER lumen and cytosolic regions for interacting with transcriptional and translational processes. Under basal conditions, the luminal domains of the ER sensors are bound by the chaperon 78-kDa glucose-regulated protein (GRP78) and as a consequence remain inactive (Frakes and Dillin, 2017). During ER-stress, unfolded or misfolded proteins accumulate and bind to the chaperons leading to their autoactivation by trans-phosphorylation (Iurlaro and Muñoz-Pinedo, 2016). Unfolded proteins can also bind directly to IRE and PERK and activate UPR by dimerization or oligomerization (Ron and Walter, 2007).

The transmembrane kinase and endoribonuclease IRE, when activated, is leading to the regulated splicing of X-box binding protein1 (*XBP1*) resulting in a frameshift generating spliced *XBP1* (*sXBP1*). SXBP1 acts as transcription factor for chaperons important for lipid synthesis and membrane expansion to help ER cope with the workload. The luminal domain of transmembrane ER-stress sensor ATF6 is bound to GRP78 under basal conditions but disrupted during ER-stress. Therefore, ATF6 is transported into the Golgi apparatus, where it is cleaved and now acts as a transcriptional factor and therefore transported into the nucleus. In the nucleus, ATF6 induces the expression of *XBP1*, C/EBP homologous protein (*CHOP)* and genes required for ERAD. PERK mainly attenuates protein translation and regulates oxidative stress. ER-stress leads to autophosphorylation and homomultimerization of the protein kinase PERK which then phosphorylates the α-subunit of eukaryotic translation initiation factor 2 (eIF2α) with its cytosolic kinase domain. It also activates the transcriptional factor nuclear factor erythroid 2-related factor 2, which induces antioxidant proteins. Other kinases such as general control non-depressible 2 and protein kinase R can also phosphorylate eIF2α. This response is known as integrated stress response and can be activated in parallel with UPR (Iurlaro and Muñoz-Pinedo, 2016). The expression of downstream transcriptional factor ATF4 depends on PERK activation and eIF2α phosphorylation

(Harding *et al.*, 2003). ATF4 also regulates *CHOP* expression. If homeostasis cannot be restored it is also the PERK branch to initiate apoptosis to protect the organisms from dysfunctional cells through activation of the PERK–eIF2α–ATF4–CHOP pathway (Wang and Kaufman, 2016).

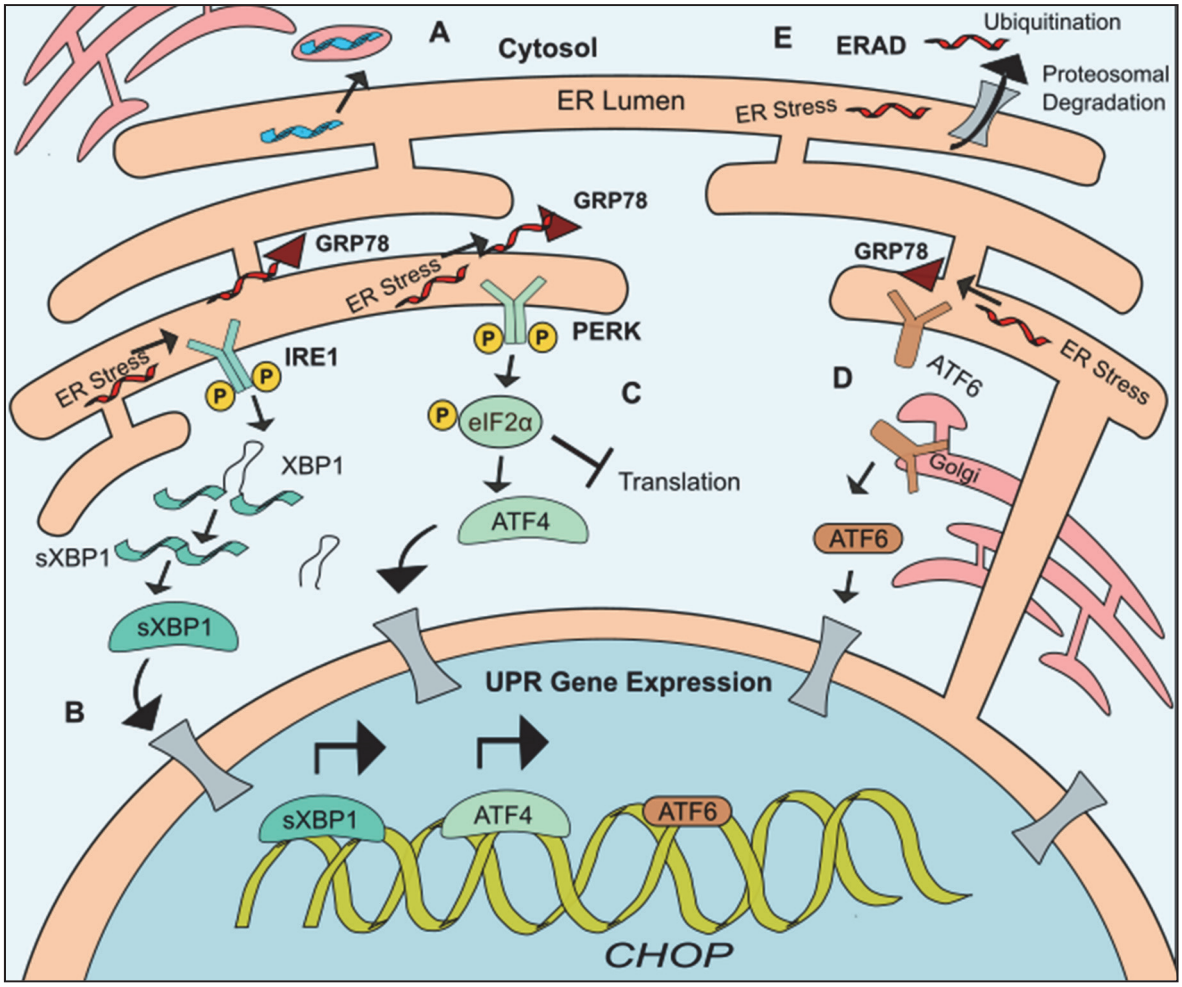

Figure 3: Schematic representation of unfolded protein response. A Nearly all polypeptides that are going to be secreted are processed in the ER lumen. Normally, the stress sensors IRE1, PERK and ATF6 are held inactive by binding to GRP78. When proteins are misfolded and accumulate in ER lumen they bind to GRP78 and releases it from the stress sensors. This activates the three main stress sensors. **B** After the phosphorylation of the dimer IRE, it is leading to the splicing of XBP1 into spliced XBP1 (sXBP1). SXBP1 then acts a transcription factor for several genes involved in UPR. **C** After homomultimerization, PERK phosphorylates the α-subunit of eukaryotic translation initiation factor 2 (eIF2α) with its cytosolic kinase domain. Phosphorylated eIF2 *α* (peIFα) increases translation of ATF4, which also regulates *CHOP* expression. **D** ATF6 is transported into the Golgi apparatus, where it is cleaved and now acts as a transcriptional factor and therefore transported into the nucleus, inducing gene expression of *XBP1*, *CHOP*, and genes for ERAD. **E** Unfolded and misfolded proteins are recognized by ERAD and transported into the cytosol for destruction by ubiquitin dependent proteasome system. Modified from Frakes and Dillin, 2017.

Abdullahi *et al.* showed that tunicamycin (TM) induces ER-stress *in vitro* in HepG2 cells, primary mouse embryonic adipocytes 3T3L1 and C3H/10T1/2 adipocytes using concentrations from 2,5 µg/mL to 20µg/mL. Compared to the ER-stress inducer thapsigargin, TM yielded a consistent heightened ER-stress response and an up- regulation of *GRP78* and *CHOP* gene expression. Cell viability

assay yielded a significant effect of high concentrations of TM. At a dose of 5µg/mL, ER stress was induced with limited adverse effect on cell viability relative to control and other doses tested (Abdullahi *et al.*, 2017).

Brefeldin A (BFA) is an ER-stress inducer produced by soil and marine fungi like *Penicillium decumbens*, which first was isolated 1958 (Paek, 2018). It has dramatic effects on mammalian cells as it inhibits some proteins that activate ADP-ribosylation factors, needed for vesicle formation. Therefore, BFA inhibits ER-Golgi transport and leads to the release of proteins into the cytosol. The accumulating proteins cause ER stress and apoptosis (Chardin and Mccormick, 1999).

1.4 The Role of ER-Stress on Pregnancy Complications

Physiological ER-stress is crucial for the development of the placenta. As reported by Iwawaki *et al.*, IRE1α-knockout mice embryos die within 12.5 days of gestation because of sever dysfunction of the placenta. Loss of IREα led to a reduction of vascular endothelial growth factor-A by half and dysfunction of the labyrinth of mice placenta, but no difference between wild type and knockout mice for HIF1-α (Iwawaki *et al.*, 2009).

In placentas of women suffering from pre-eclampsia, the fail of transformation from cytotrophoblast with an epithelial phenotype to an endothelial phenotype due to a lack in the expression of integrin, cadherin and Ig subfamily members has been reported (Zhou, Damsky and Fisher, 1997). This defective spiral artery remodeling may cause an imbalance between antioxidant and pro-oxidant mechanisms leading to oxidative stress at the maternal-fetal interface. This can be supported by *in vitro* studies showing increased levels of reactive oxygen species (Phipps *et al.*, 2019). ER-stress was reported in studies of placental tissues of women with pre-eclampsia. Fu *et al*. compared expression of ER-stress markers in placentas of early onset and late onset severe pre-eclampsia to a control group. A significant increase in PERK signaling pathway ER-stress markers was found in both groups compared to control group, indicating that ER-stress-induced apoptosis plays an important role in the development of severe pre-eclampsia by activation of PERK signaling pathway induced apoptosis (Fu *et al.*, 2015).

Gestational diabetes mellitus (GDM) is described as a state with elevated glucose level, increased insulin resistance and higher levels of oxidative stress. Yung *et al*. report that ER-stress is not caused by the high glucose level but the

result of lactic acid in in vitro studies with BeWo-NG cell line (Yung *et al.*, 2016) and refer to Taricco *et al*. reporting a significant increase of lactate concentration using indirect measures in the umbilical vein in GDM pregnancies (Taricco *et al.*, 2009). Oxidative stress can also lead to ER-stress in the choriocarcinoma cell line JEG-3 (Yung *et al.*, 2008). This suggests that intracellular glucose deprivation and high serum glucose levels lead to increased lactic acid concentration and oxidative stress, which in turns promote misfolded or unfolded proteins that induce ER-stress (Guzel *et al.*, 2017).

1.5 Insulin in Pregnancy

As mentioned above, an increased insulin resistance is found in GDM. Insulin is an anabolic hormone produced in the β-cells of the pancreas and secreted into the portal circulation. On its way to the liver, 50% of insulin is cleared by hepatocytes. The rest of the insulin exits the liver and is transported via hepatic vein to the heart to be distributed by arterial circulation to the body and its effector organs such as muscles and fat cells. There it stimulates GLUT4 translocation and glucose uptake. Insulin is a pleotropic factor with multiple effects. In the liver, it suppresses gluconeogenesis and glycogenolysis and, therefore, regulates blood glucose level. It also has an vasodilating effect in endothelial cells of arteries and arterioles by initiating NO production (Tokarz, MacDonald and Klip, 2018). During pregnancy, β-cell adaption of the mother takes place to handle the changing needs of mother and fetus from early to late pregnancy (Baeyens *et al.*, 2016). In normal pregnancy insulin sensitivity decreases from early (0.7x) to late (0.4x) pregnancy accompanied by an decrease in fasting glucose level in late pregnancy (0.9x)(Lain and Catalano, 2007).

Insulin can also induce ER-stress in different tissues. For example in non-diabetic subjects a raise in serum insulin level leads to an increase in UPR mRNA and proteins and modulates induction of GRP78 during ER-stress (Inageda, 2010; Boden *et al.*, 2014).

A toxic effect of pathologic insulin concentrations have been reported in first trimester trophoblast cells, isolated of human placentas by analyzing DNA damage markers as well as increased cell survival compared to vehicle control under conditions of 21% O_2 (Vega, Mauro and Williams, 2019). Studies in first trimester cell line HTR8/SVneo also showed an antiproliferative and hypertrophic effect of

insulin but no significant effect on cell viability and apoptosis (Silva *et al.*, 2017) also under conditions of 21% O_2. However, little is known about the effect of insulin on ER-stress in first trimester placenta under physiological incubation conditions.

1.6 *In Vitro* Placenta Models in Literature

Animal models for studying human placenta development and its different aspects such as endocrine functions, trophoblast differentiation and immunology have their limitations. For this reason, there are numerous in-vitro cell line and tissue models as mentioned above. Placenta explants have been used early on to study oxygen demand, transport of amino acids and small molecules in adhered or floating cultures. Explants enable to study the differences between physiological and pathological placenta tissue of same age. Because of the different cell types in placenta explants, they are usually used to study functions of trophoblasts. In comparison with primary trophoblasts, trophoblasts in placenta explants do not undergo stressful isolation protocols and remain in a physiological state. Depending on gestational age, placenta explants can be cultured at different O_2 tensions up to 12 days in Hypoxia workbenches. For studying of angiogenesis endothelial cells of the placenta or human umbilical vein endothelial cells can be isolated and cultured in two- or three-dimensional cultures (Huppertz and Schleußner, 2018).

Most primary trophoblast isolation procedures are based on Kliman's method for villous placenta tissue from the 1980's, which have been improved over the time to increase purity of the trophoblast population. These methods for isolation of primary trophoblasts play an important role for studying pathologies as gestational diabetes and preeclampsia. There are also disadvantages of primary trophoblast cultures such as limited availability of placenta tissue of the first trimester, the variability between samples, low harvest and low cell division rate. On the contrary, trophoblast cell lines are a homogenous cell population, show a stable phenotype and can be cultured for many passages. There are three different groups of trophoblastic cell lines; choriocarcinoma cell lines, trophoblast hybridoma cell lines and transformed trophoblasts, which differ in gene expression compared to primary trophoblasts. Trophoblast hybridoma cell lines combine phenotypic characteristics of primary trophoblasts with the advantages of choriocarcinoma cell lines (Hiden *et al.*, 2007). For example, ACH-1P cell line are primary trophoblasts fused with hypoxanthine-guanine-phosphoribosyl- transferase (HGPRT)- defective mutant

choriocarcinoma clones (Frank *et al.*, 2000). Another hybrid cell line is ACH-3P, which expresses trophoblast specific marker, was developed by fusing AC1-1 cell line with first trimester trophoblasts and, therefore, a robust model to study the first trimester of human pregnancy (Hiden *et al.*, 2007; Huppertz and Schleußner, 2018).

1.7 Hypothesis and Objective

As described above, insulin plays an important role in pregnancy as an increased insulin resistance and hyperinsulinemia is reported in adverse pregnancy outcomes and has an impact on future health of mother and child.

Insulin is also known to regulate ER-stress in different human tissues such as adipose tissue and neuroblastoma cells (Boden et al., 2014; Inageda, 2010).

I hypothesized that ER-stress can be induced in ACH-3P cells under physiological oxygen concentration and that insulin is able to decrease this effect.

This thesis had the objective to determine the effect of insulin, as well as the effect of insulin in combination with ER-stress inducers on UPR in the first trimester trophoblast cell line ACH-3P.

2 Material and Methods

2.1 ACH-3P Cell Culture Model

Cryo-conserved ACH-3P cells were used for cultivation in 75 cm² flasks (Thermo Fisher Scientific, Roskilde, Denmark). Cultivation medium consisted of Ham's F-12 Nutrient Mix+ L-Glutamine medium (Thermo Fisher Scientific) supplemented with 10% (v/v) fetal bovine serum (FBS, Cytiva, USA) and 1% (v/v) Penicillin/Streptomycin (Thermo Fisher Scientific) to prevent contamination. Firstly, cells were incubated at 37°C in an atmosphere containing 21% O_2 and 5% CO_2. Medium was changed every 3-4 days. Cells were split when they reached around 80%-90% confluence.

For splitting, 4 mL Accutase solution (Merck, Darmstadt, Germany) was added for removing cells enzymatically from the flasks. After incubation for 8 minutes at 37°C, the reaction was stopped by adding 6 mL fresh incubation medium followed by centrifugation (10 min, 1200 rpm, 4°C). Following resuspension of the cell pellet in fresh medium by pipetting, the cell solution was pipetted through a 70µm cell strainer (Corning Incorporated, Durham, USA) to remove aggregates for counting the cell number with CASY Cell Counter and Analyzer (OMNI Life Science, Bremen, Germany).

For each experiment, 25.000 cells were seeded onto each well of seven 6-well plates. Cell density was checked with EVOS XI Core microscope.

To mimic the physiologically low intrauterine oxygen tension during the first trimester, experiments were conducted at an oxygen tension of 6.5% O_2 and 37°C in XVIVO System hypoxia cell culture hood (BioSpherix, Redfeld, USA).

ACH-3P cells were incubated once for 24 hours with selection medium to avoid overgrowth of carcinoma cells containing Ham's F-12 supplemented with 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin, 25mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES, Thermo Fisher Scientific), 0.1% glucose (Merck), 5.7µM azaserine and 100µM hypoxanthine (Hybri-Max™ Azaserine-Hypoxanthine, Merck). Hiden et al. recommend the use of selection medium every two passages (Hiden *et al.*, 2007) and laboratory protocol every five passages.

2.2 Treatments and Experimental Set-Up

For each experiment, the same concentrations were used. A control without treatment was added. For insulin treatment a pathologic insulin concentration of 10nM was chosen based on previous experiments with ACH-3P cells and literature research (Westgate *et al*. 2006).

TM and BFA were used to induce ER-stress in cells. Conditions were tested with and without 10nM insulin. TM was used in a final concentration of 3µg/mL and BFA concentration of 10µg/mL.

Each used solution for any kind of treatment of cells in hypoxia cell culture hood was incubated minimum two hours under hypoxia conditions (6.5% O_2, 37°C) before use to avoid influence by higher O_2 tension. Each condition was assayed in triplicates and two times, one for protein analysis by immunoblotting and one for RNA for RT-qPCR (*Figure 4*).

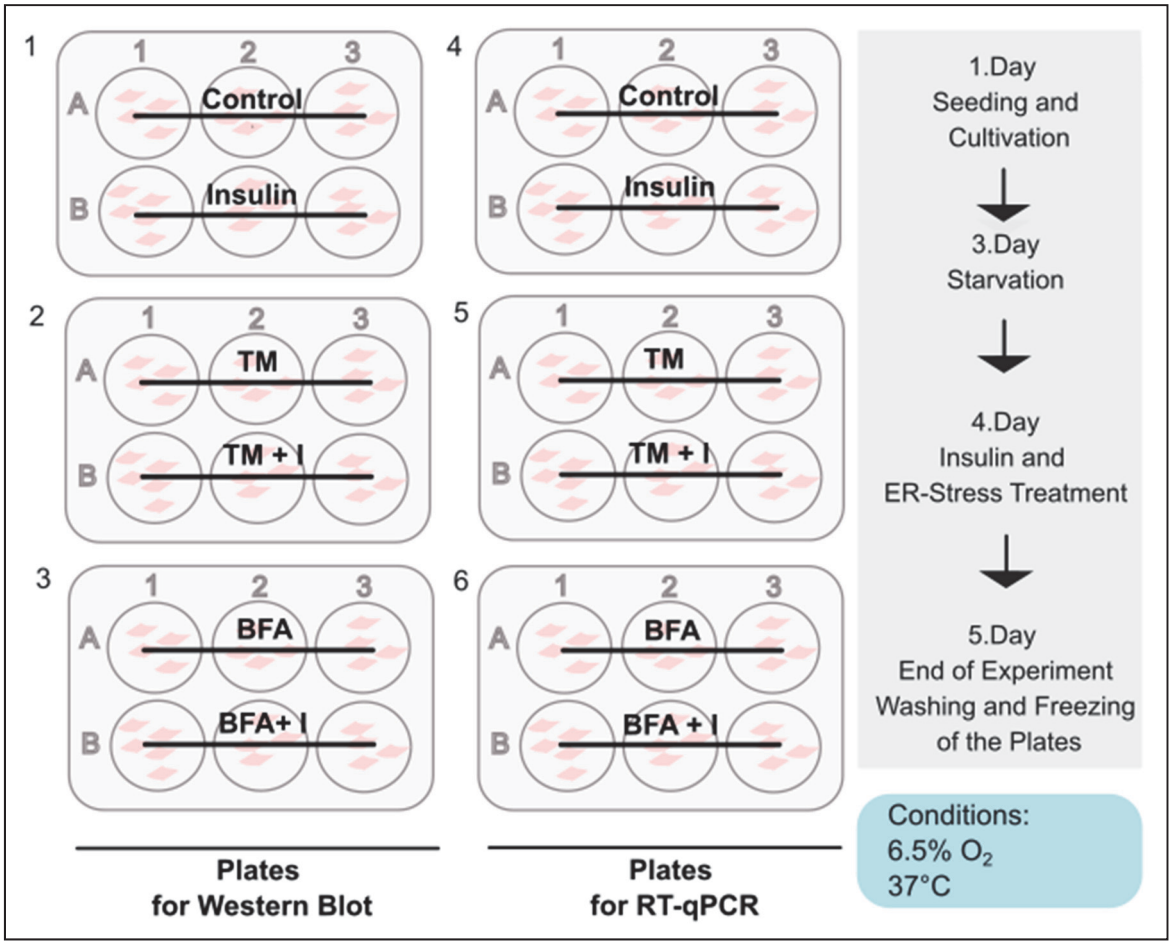

Figure 4: Experimental set-up.

Seeded cells were cultivated for 48 hours in hypoxia cell culture hood before serum starvation. After 24 hours of serum starvation, cells were washed with Hank's Balanced Salt Solution (HBSS, Gibco, Thermo Fisher Scientific). Insulin, BFA + insulin, TM and TM + insulin dilutions were freshly prepared for each treatment. Cells were incubated 24 hours under 6.5% oxygen tension in hypoxia cell culture hood before removing supernatant and washing with HBSS. Plates were frozen immediately at -20°C for storing.

2.3 Protein Isolation

Six-Well plates were thawed on ice. Total protein was isolated using 60µl of cold RIPA buffer supplemented with proteinase inhibitors (Complete Mini Protease-Inhibitor Cocktail, Roche) for each well. After cell scraping, lysates were centrifuged for 20 min at 13,000 rpm and 4°C and the supernatants were collected on ice.

Protein concentration was measured in duplicates using the bicinchoninic acid assay (BCA) (Thermo Fisher Scientific). After 30 min of incubation at 37°C absorbance was measured using the SPECTROstar Omega absorbance microplate reader.

2.4 Western Blot

The protein lysates were diluted in 4x concentrated Laemmli buffer (Bio-Rad, Hercules, CA, USA) and denatured for 5 min at 95°C (Thermomixer comfort, Eppendorf). Total protein (5µg; loading volume 5-29.3µl) were loaded onto 4-20% Mini-PROTEAN precast TGX gradient gels (Bio-Rad). Electrophoresis was performed at 120 V, 400 mA for 70 min. PageRuler Protein Ladder (Thermo Fisher Scientific) (10-170kDa) was used as molecular weight markers. Protein lysate of ACH-3P cells cultivated in a flask and 6.5% O_2 tension was loaded onto every gel and used as a calibrator for normalization between membranes.

For blotting the proteins onto a nitrocellulose membrane (Bio-Rad), the Trans-Blot Turbo System (mixed molecular weight, 7 min, BioRad) was used. Quality of the electrophoresis and protein transfer was assessed by Ponceau S staining (Sigma-Aldrich). After washing with distilled water, membranes were incubated on a shaker for one hour with a blocking solution (Pierce™ Clear Milk Blocking Buffer, Thermo Fisher Scientific) to block non- specific bonding sites.

For detection and quantification of proteins, first phosphorylated proteins

were quantified by incubation with primary antibodies and secondary antibodies for signal enhancement. Membranes were incubated overnight at 4°C with primary antibodies (Table 1) on a shaker followed by washing for 1h with TBE + 0.2% Tween and incubation for 1h at RT with the corresponding secondary antibody. Before quantification of non- phosphorylated proteins, Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) was used to remove antibodies against the phosphorylated proteins.

After 1h- washing with TBE + 0.2% Tween, SuperSignal West Pico PLUS solution as chemiluminescent substrate (Thermo Fisher Scientific) and ChemiDocTouch (Bio-Rad) were used to visualize the immunolabelling. Quantification of band densities was performed with Image Lab software (Bio-Rad).

Primary antibody	Molecular weight target	Host	Dilution primary antibody	Dilution secondary antibody
pIRE1α	110kDa	rabbit	1:1000 (5% BSA)	1:2000
IRE1α	110kDa	rabbit	1:1000 (5% BSA)	1:2000
peIF2α	38kDa	rabbit	1:1000 (5% BSA)	1:2000
eIF2α	38kDa	mouse	1:1000 (5% BSA)	1:2000
GRP78	78kDa	mouse	1:5000 (5% Milk)	1:5000
α-tubulin	55kDa	mouse	1:1000 (5% Milk)	1:5000

Table 1: Primary antibodies used for immunolabeling.

2.5 RNA Isolation and cDNA Synthesis

ACH-3P total RNA was isolated using the miRNeasy Mini Kit (Qiagen, Germany) as per manufacturer's guidelines. RNA concentration was measured with the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). For reverse transcription of RNA to complementary DNA (cDNA), LunaScript RT SuperMix Kit (New England BioLabs, Ipswich, MA, USA) and a PCR thermocycle (Bioanalytica) were used. After cDNA synthesis 180µl RNase free water were added to the 20µl samples to a final concentration of 2.5 ng/µl (500ng in 200µl volume) as template for TaqMan RCR.

2.6 Quantitative Real-Time PCR

For quantitative real-time polymerase chain reaction (RT-qPCR) a TaqMan Universal PCR Master Mix (Applied BiosystemsTM, Thermo Fisher Scientific) and

a TaqMan gene expression assays and the CFX384 Thermocycler (Bio- Rad) were used**.** Ten ng of cDNA per reaction were used in Hard-Shell® 384-well PCR plates (Bio-Rad). Peptidyl-prolyl-isomerase A (*PPIA*) and TATA-box binding protein (*TBP*) were used as housekeeping genes.

For analysis, cycle threshold (Ct) values were used for calculation of ΔCt values for every target gene by subtraction of the mean of housekeeping genes Ct value from Ct value of target gene. Splicing of *XBP1* was determined by subtracting Ct values of *XBP1* from CT value of *sXBP1.*

Gene	Taqman gene assay	Fluorophore label
CHOP	Hs000358796_g1	FAM
XBP1	Hs02856596_m1	FAM
sXBP1	Hs03929085_g1	FAM
TBP	Hs00427620_m1	VIC
PPIA	Hs04194521_s1	VIC

Table 2: Gene expression assays used for RT-qPCR.

Step	Duration	Temperature
Pre-incubation	00:02:00	50°C
Initial denaturation	00:10:00	95°C
Denaturation	00:00:15	95°C
Annealing	00:01:00	60°C

Table 3:RT-qPCR temperature profile.

Four different passages of ACH-3P cells have been analyzed. Immunoblot and RT-qPCR results have been statistically evaluated with GraphPad Prism (GraphPad Prism 5 for Windows, 2007). Distribution of raw data was assessed and unpaired t-test was used for normal distributed data and Mann-Whitney U test for non-normal distribution.

3 Results

3.1 Effects of Insulin on GRP78 Protein Levels in ACH-3P Cells

GRP78 is regulating ER-stress initiation mediators IRE1α, ATF6, and PERK. GRP78 protein was determined by western blotting and results were normalized to α-tubulin. Results for GRP78 showed no significant difference between control samples and insulin treated cells. When compared to control, treatment with TM and BFA induced a significant upregulation of GRP78 protein levels (8.6-fold and 4.7-fold, respectively; p≤0.001). No difference could be found between TM and TM+I as well as between BFA and BFA+I *(Figure 5).*

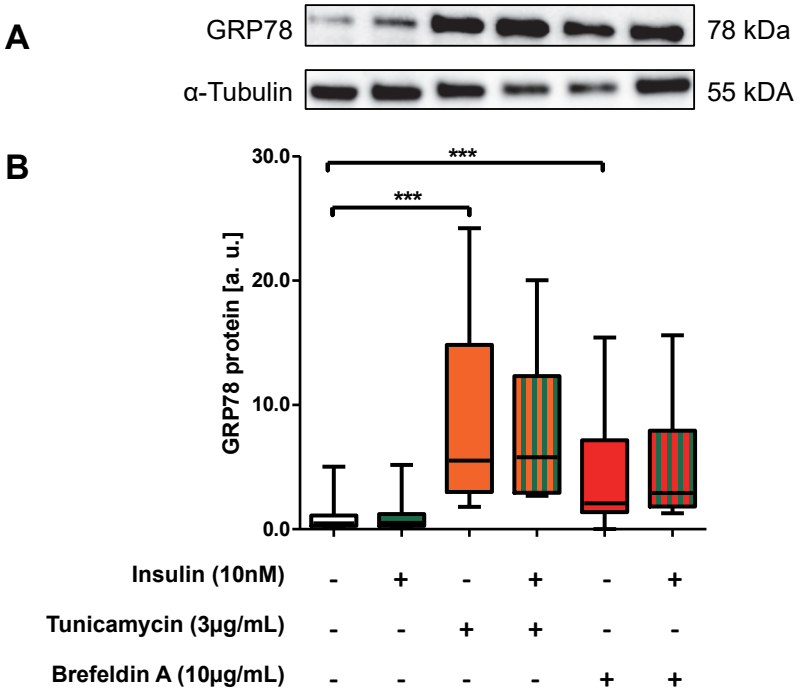

Figure 5: Effect of Insulin and ER-stress inducers on GRP78. A Representative western blot for GRP78. ACH-3P cells were cultured for 24 hours and 6.5% O_2 in absence or presence of insulin (10nM), tunicamycin (3µg/mL), brefeldin A (10µg/mL) or their combination and quantified by western blot using α-tubulin for normalization. **B** Statistical analysis of western blots for GRP78. Statistical analysis was performed using t-test and Mann-Whitney-U-test. (n=4) ***p≤0.001

3.2 ER-Stress Inducers Increase IRE1α Protein

The release of GRP78 from ER-stress sensor IRE1α leads to autophosphorylation of IRE1α to pIRE1α, which triggers the splicing of *XBP1*. Spliced *XBP1* acts as transcriptional factor for the expression of important genes involved in UPR (Frakes and Dillin, 2017).

Total IRE1α protein as well as phosphorylation of IRE1α was determined. Western blot analysis showed no difference between control and insulin treated cells for IRE1α. An increase of IRE1α between control and TM and BFA treated cells could be shown (2.2-fold and 1.9-fold, respectively; p≤0.01) but no difference between cells incubated with the ER-stress inducers alone and ER-stress inducers in combination with insulin *(Figure 6).*

When comparing phosphorylated to non-phosphorylated IRE1α protein, less phosphorylation was observed with ER-stress inducers TM and BFA compared to control (0.5-fold and 0.4-fold, respectively; p≤0.05) (*Figure 7*).

XBP1 expression and its splicing were measured using RT-qPCR and results were normalized to the mean expression of housekeeping genes PPIA and TBP.

Relative expression of *XBP1* was significantly increased in cells treated with insulin (p≤0.01) (*Figure 8*), but no effect of insulin could be detected on *XBP1* splicing. Both ER-stress inducers increased relative expression of *XBP1* (1.7-fold and 1.6-fold, respectively; p≤0.0001 and p≤0.05), but again no difference between cells treated only with ER-stress inducers compared to additional insulin treatment. ER-stress inducers TM and BFA significantly increased *XBP1* splicing (8.5-fold and 12-fold, respectively; p≤0.001) (*Figure 9*).

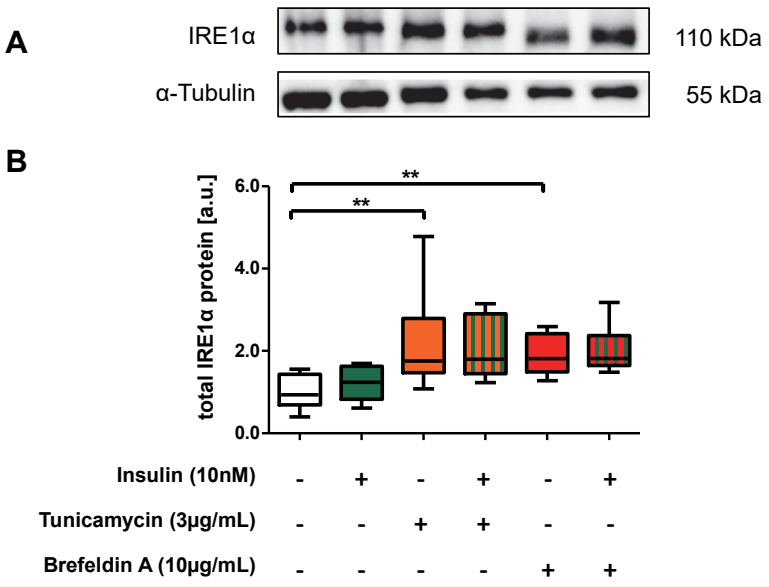

Figure 6: Results for total IRE1α. A Representative Western blot for total IRE1α. ACH-3P cells were cultured for 24 hours and 6.5% O_2 in absence or presence of insulin (10nM), tunicamycin (3µg/mL), brefeldin A (10µg/mL) or their combination and quantified by western blot using α-tubulin for normalization **B** Statistical analysis of western blots for total IRE1α. Statistical analysis was performed using t-test and Mann-Whitney-U-test (n=3), ** p<0.01

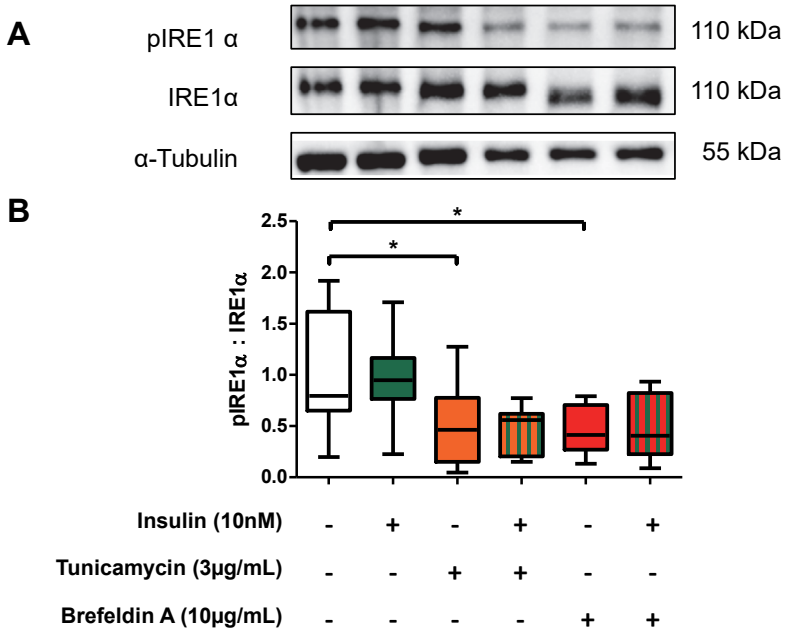

Figure 7: Results for IRE1α phosphorylation. A Representative western blot for relation of IRE1α phosphorylation. ACH-3P cells were cultured for 24 hours and 6.5% O_2 in absence or presence of insulin (10nM), tunicamycin (3µg/mL), brefeldin A (10µg/mL) or their combination. IRE1α and phosphorylated IREα were quantified by western blot using α-tubulin for normalization **B** Statistical analysis was performed using t-test and Mann-Whitney-U-test for IRE1α phosphorylation (n=3). * p< 0.05

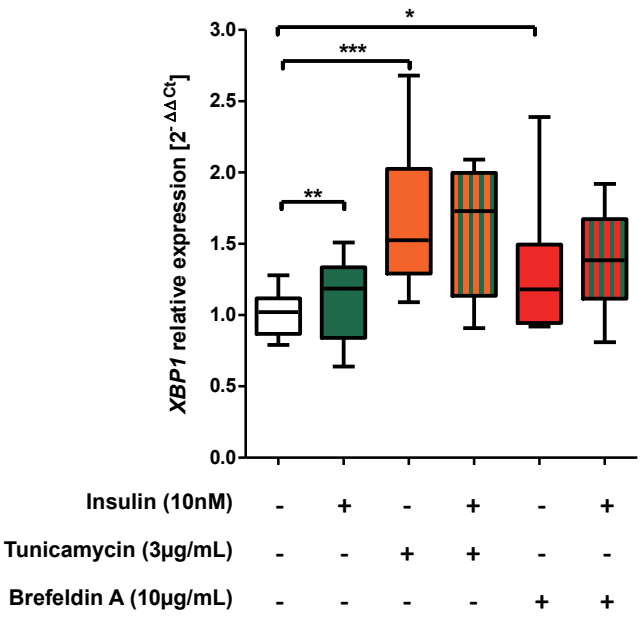

Figure 8: Relative expression of *XBP1*. ACH-3P cells were cultured for 24 hours and 6.5% O_2 in absence or presence of insulin (10nM), tunicamycin (3µg/mL), brefeldin A (10µg/mL) or their combination. Relative expression of *XBP1* was analyzed by RT-qPCR and normalized to the mean expression of *TBP* and *PPIA*, used as housekeeper genes. Statistical analysis was performed using t-test and Mann-Whitney-U-test (n=4) *** p<0.001, **p<0.01, * p< 0.05

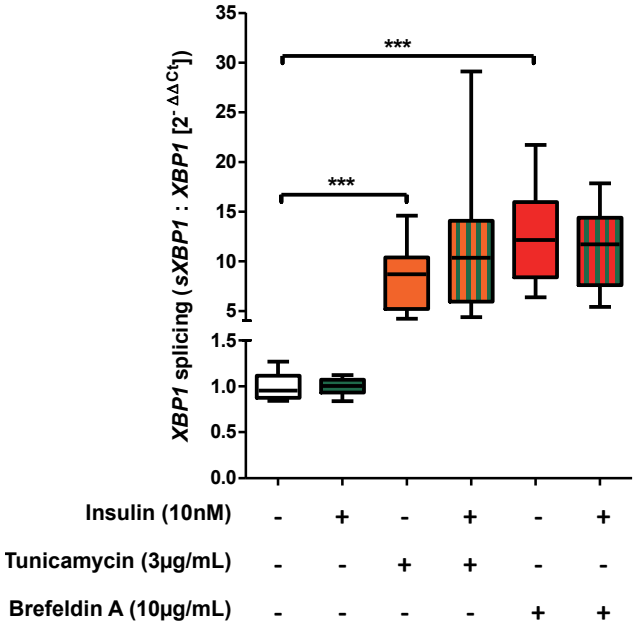

Figure 9: Effect of ER-stress inducers on *XBP1* splicing. ACH-3P cells were cultured for 24 hours and 6.5% O_2 in absence or presence of insulin (10nM), tunicamycin (3µg/mL), brefeldin A (10µg/mL) or their combination. Splicing of XBP1 was determined by subtracting Ct values of *XBP1* from Ct value of *sXBP1*. Statistical analysis was performed using t-test and Mann-Whitney-U-test (n=4) *** p<0.001

3.3 Effect of Insulin on PERK Pathway

PERK is a protein kinase, phosphorylating eIF2α subunit, which leads to translation of ATF4, a regulator of various genes such as *CHOP* (Frakes and Dillin, 2017). Therefore, eIF2α protein as well as phosphorylated eIF2α were quantified and *CHOP* expression was analyzed. In contrast to IRE1α, no difference between groups for eIF2α protein and phosphorylation with ER-stress inducers TM and BFA was found *(Figure 10).* On the other hand, *CHOP* expression was increased comparing control to TM and BFA treated cells (4.8-fold and 4.9-fold, respectively; p≤0.001), indicating ER-stress signaling by eIF2α pathway. In concordance with previous results presented insulin had no effect on ER-stress inducer treated cells *(Figure 11).*

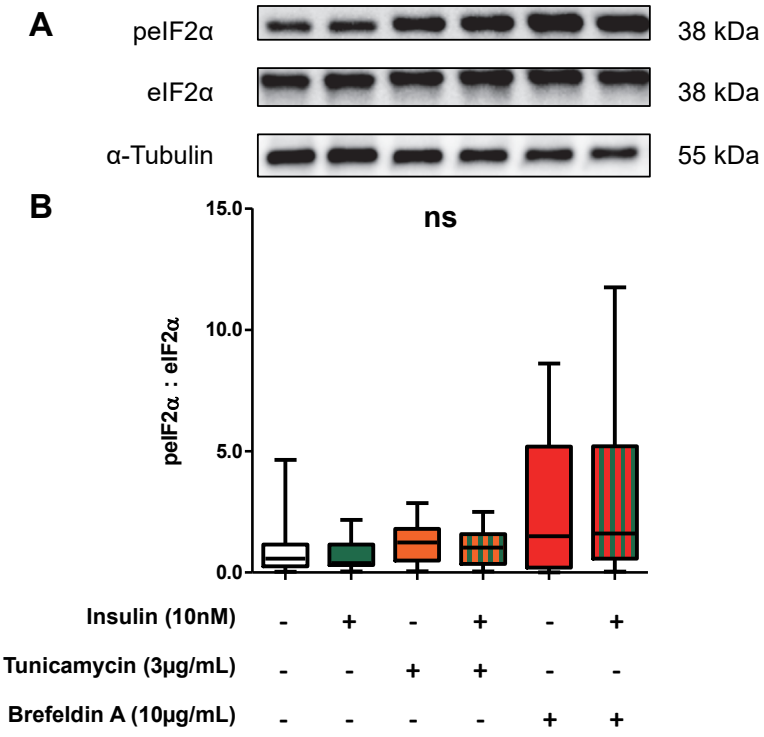

Figure 10: Effects of insulin and ER-stress inducers on eIF2α phosphorylation. A Representative western blot for peIF2α and eIF2α. ACH-3P cells were cultured for 24 hours and 6.5% O_2 in absence or presence of insulin (10nM), tunicamycin (3µg/mL), brefeldin A (10µg/mL) or their combination. EIF2α and phosphorylated eIF2α were quantified by western blot using α-tubulin for normalization **B** Statistical analysis of eIF2α phosphorylation was performed using t-test and Mann-Whitney-U-test (n=4). ns = not significant

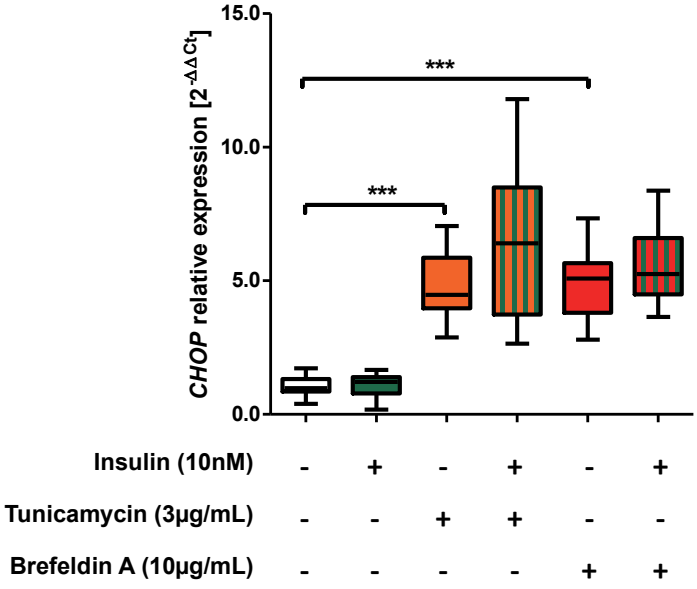

Figure 11: Relative *CHOP* expression. ACH-3P cells were cultured for 24 hours and 6.5% O_2 in absence or presence of insulin (10nM), tunicamycin (3µg/mL), brefeldin A (10µg/mL) or their combination. Relative expression of *CHOP* was analyzed by RT-qPCR and normalized to the mean expression of *TBP* and *PPIA*, used as housekeeper genes. Statistical analysis was performed using t-test and Mann-Whitney-U-test (n=4) *** $p<0.001$

4 Discussion

Physiological ER-stress is an important driver of angiogenesis of the fetal placenta (Patel *et al.*, 2010). Therefore, it is crucial to an adequate supply for the growing fetus. In contrast, an increase of ER-stress is reported in pregnancy complications like pre-eclampsia and GDM (Yung *et al.*, 2008). As previously mentioned, insulin has an ER-stress regulating effect in adipose tissue and neuroblastoma cells, but little is known about the effect of insulin on ER-stress in first trimester placenta (Boden et al., 2014; Inageda, 2010).

ACH-3P cells have been cultivated and incubated for 24 hours with 10nM insulin and with 3µg/mL TM or 10µg/mL BFA alone or in combination with insulin. ER-stress markers and sensors have been measured and compared to control. The present study was not able to detect any ER-stress regulating effect of insulin. However, the ER-stress markers GRP78, IRE1α, *CHOP* and splicing of *XBP1* were increased in ER-stress inducer treated cells. This suggests that TM and BFA can be used in ACH-3P cells for ER-stress induction. Phosphorylation of IRE1α was decreased with ER-stress inducers. This finding may be the result of phosphatase activity (Bononi *et al*., 2011) overriding the phosphorylation effect on IRE1α at the experimental time point.

Although no difference between the treatments when analyzing total eIF2α protein could be shown, increased *CHOP* expression indicates ER-stress induction. This might be due to activation of *CHOP* expression by other factors like ATF6 and *XBP1* (Iurlaro and Muñoz-Pinedo, 2016). Missing increase of eIF2α may be a time point dependent effect. For example, ER-stress analysis in JEG-3 cells show that UPR can be activated separately and at different levels of ER-stress with tunicamycin (Burton *et al*., 2009)

Preliminary results led to the conclusion that passage number has an impact on the results and so does possibly the use of selection medium between passages. As mentioned before, ACH-3P should be treated every two passages with selection medium to prevent the overgrowth of choriocarcinoma cell phenotype. The relation between first trimester and choriocarcinoma cell phenotype could lead to altered and wide distributed data. To investigate this possibility, pre and post selection medium results for GRP78, splicing of *XBP1*, total IRE1α and *CHOP* were split and visualized with boxplots (App.Figure 1-4). Because of a small number of samples

when split, no statistical analysis could be performed. A trend can be seen in App.Figure 3. Data values are more spread in pre-selection medium use results for total IRE1α protein.

ACH-3P was used to assess if insulin has an ER-stress regulating effect in early pregnancy placenta. One of the major strengths of this study is the cultivation of first trimester trophoblast cell line under, for early placenta conditions, physiological O_2 tension of 6.5%.

However, future studies should cover different insulin and ER-stress inducer concentration, but also include passage number into analysis or use low passage number to prevent choriocarcinoma phenotype overgrowth.

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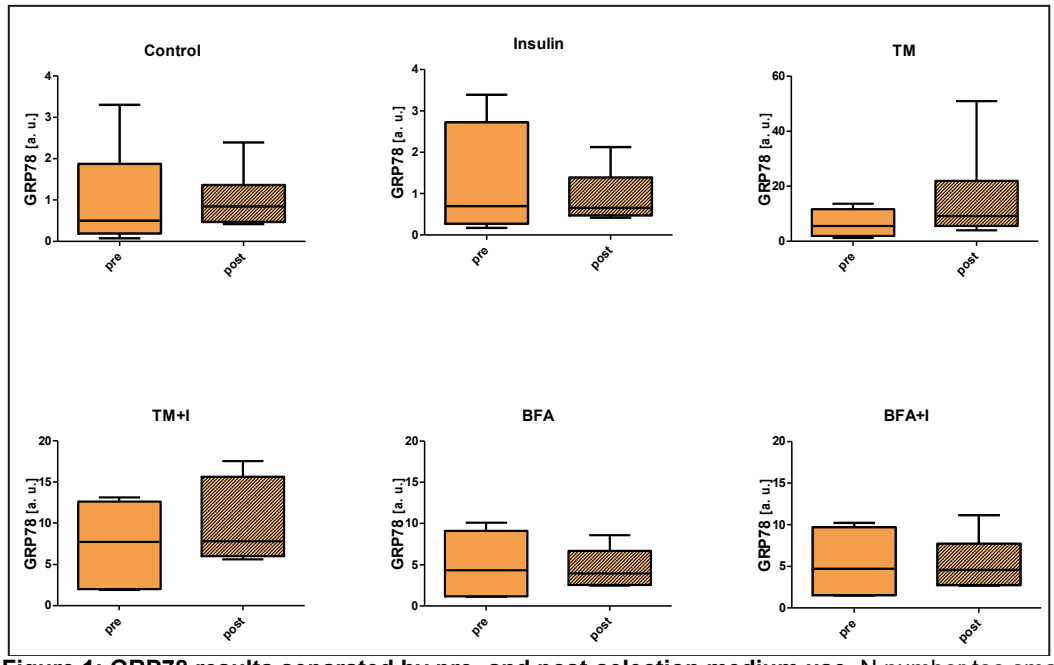
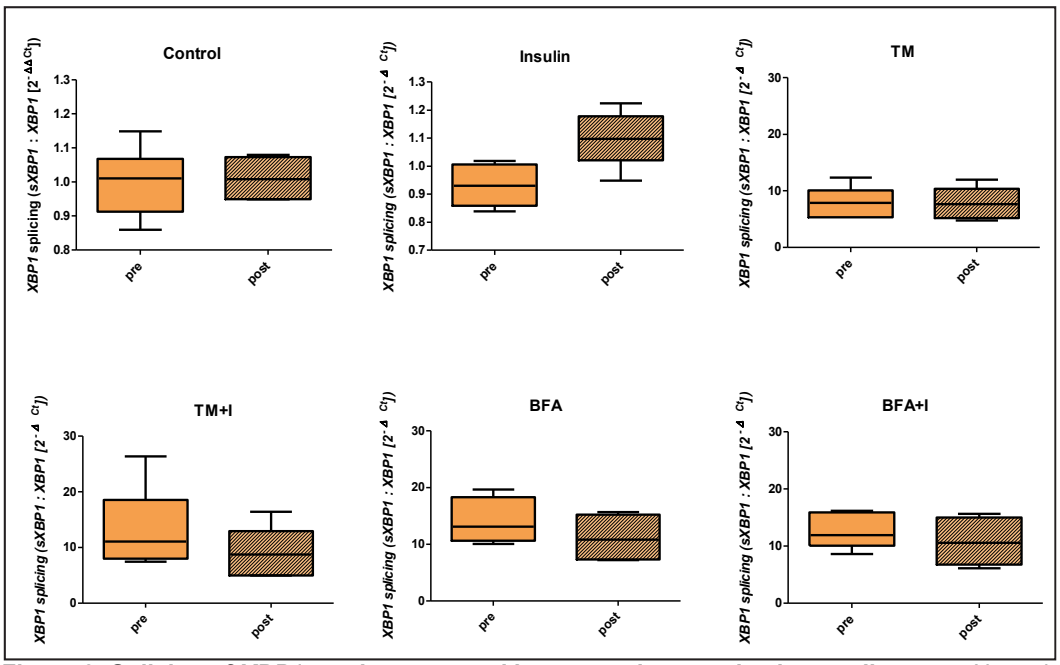
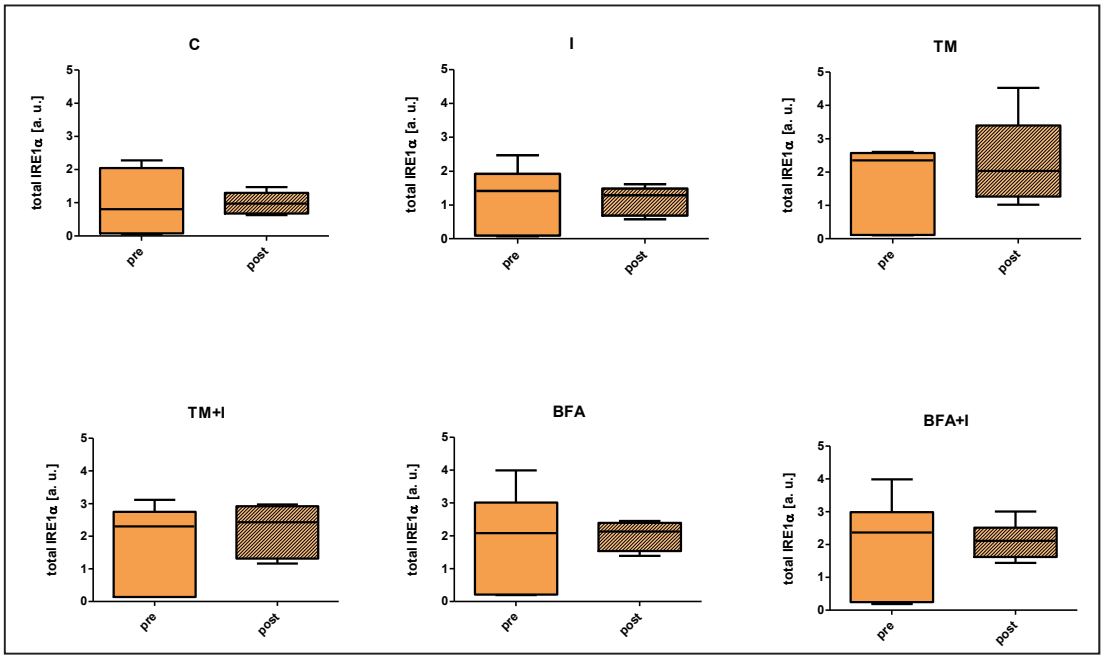
6 Appendix



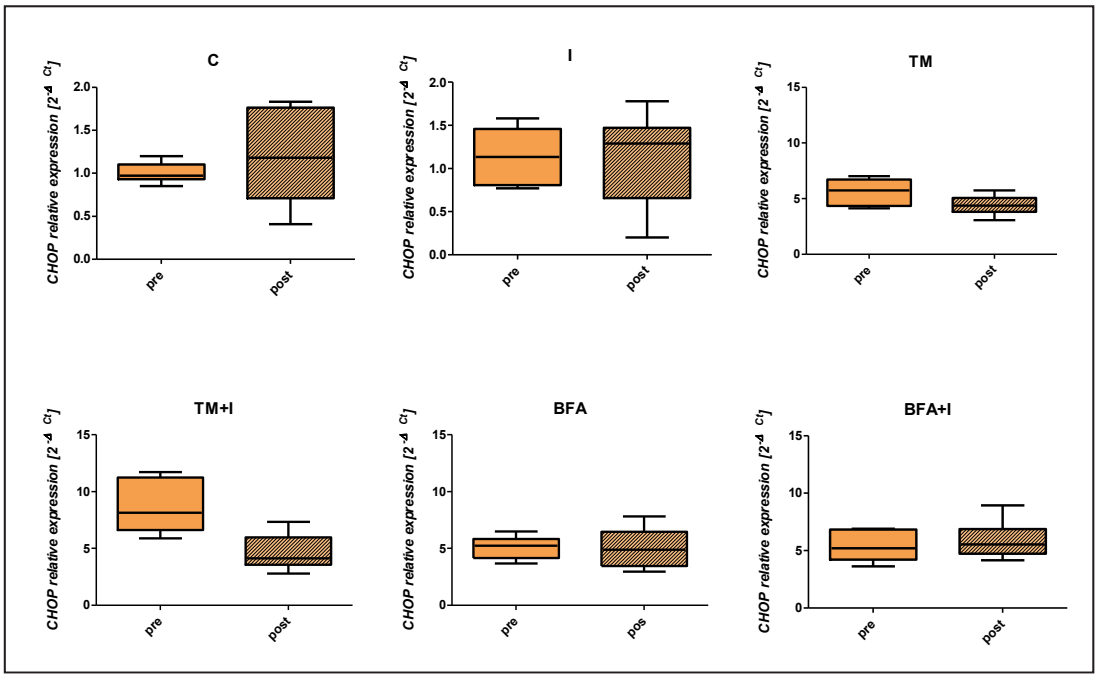
App. Figure 1: GRP78 results separated by pre- and post-selection medium use. N number too small for statistical analysis. (n=2) (pre-selection medium: orange bar, post-selection medium: hatched bar)



App. Figure 2: Splicing of *XBP1* results separated by pre- and post-selection medium use. N number too small for statistical analysis. (n=2) (pre-selection medium: orange bar, post-selection medium: hatched bar)



App. Figure 3: Total IRE1α results separated by pre- and post-selection medium use. N number too small for statistical analysis. (n=2) (pre-selection medium: orange bar, post-selection medium: hatched bar)



App. Figure 4: Relative expression of *CHOP* results separated by pre- and post-selection medium use. N number too small for statistical analysis. (n=2) (pre-selection medium: orange bar, post-selection medium: hatched bar)