Infertility affects a high percentage of the mature population. However, the success rate of assisted reproduction (ART) is only 30%. The current goal of ART is the safe transfer of a healthy, viable, single embryo. Accurate and rapid methods of quantifying embryo viability are needed to reach this goal. Molecular markers are still weakly incorporated into clinical practice. Methodological advances (e.g., the invasive preimplantation genetics) have the potential to make an important contribution, however, there has been a drive to develop alternative non-invasive methods to better meet clinical needs. Metabolic profiling of spent embryo culture (SEC) media should offer an exceptional opportunity for the assessment of embryo viability. Using LC-MS we have successfully identified a fragment of the human haptoglobin molecule, which largely differed in quantity in SEC between viable and non-viable embryos. Morphological assessment of the embryos along with the HPLC coupled high-resolution MS measurement of SEC is part of the current project. Rapid measurement of the haptoglobin fragment from SEC with a simple bedside method would be of obvious clinical interest. Implementation of lab-on-a-chip technology (chip surface chemistry) is planned. PCR-based short tandem repeat methodology and characterization of cftDNA also remains a challenging task. Next generation sequencing is revolutionizing genetic research and diagnostics; however, this technique has never been adapted to the analysis of SEC. Another goal is the confirmation of actively secreted miRNA fractions from total cfmiRNA in human SEC and a comparative analysis of cfmiRNA fractions from the mouse embryo and SEC.

1. The impact of tryptophan-kynurenine and tryptophan-serotonin (5-HT) pathways on reproductive performance during IVF:

A cross-sectional clinical study was designed in 64 consecutive IVF patients. The analysis was done by using LC-MS/MS. In IVF patients ovarian hyperstimulation results in a reduction of the availability of tryptophan to catabolic pathways to kynurenine and 5-HT. Outcome measures improved significantly when 5-HT predominated over kynurenine. Another study was performed to examine the effect of interactions between serotonin (5-HT), brain-derived neurotrophic factor (BDNF), and kisspeptin on the reproductive potential in women receiving IVF. Serum and FF 5-HT, BDNF, kisspeptin, and platelet-activating factor (PAF) levels were measured by enzyme-linked immunosorbent assay. In response to ovarian hyperstimulation, serum 5-HT and kisspeptin levels significantly increased, whereas serum BDNF and PAF levels remained unchanged. FF 5-HTand BDNF levels were positively correlated. Serum kisspeptin levels were negatively correlated with FF BDNF and serum and FF PAF levels. Women who were pregnant had significantly lower FF BDNF levels compared with women who were not pregnant. Multivariate stepwise linear regression and logistic regression analyses showed that only 5-HT and kisspeptin improved IVF outcome. This study indicates a role of serotoninergic mechanisms in success of IVF. An observational, clinical study was designed to assess the role of sirtuin 1 (SIRT1), sirtuin 6 (SIRT6), and resveratrol in IVF. Ovarian hyperstimulation resulted in significantly higher serum SIRT1 levels in pregnant women (8 patients) compared with non-pregnant women (22 patients). SIRT6 levels remained unchanged after ovarian

hyperstimulation but were significantly lower in pregnant women compared with non-pregnant women before and after hyperstimulation. Both SIRTs were detected in FF, but they appeared to be independent of their serum levels. Our study shows that SIRT1 and SIRT6, but not resveratrol, are involved in human reproduction and they may have a role in oocyte maturation and clinical pregnancy in IVF.

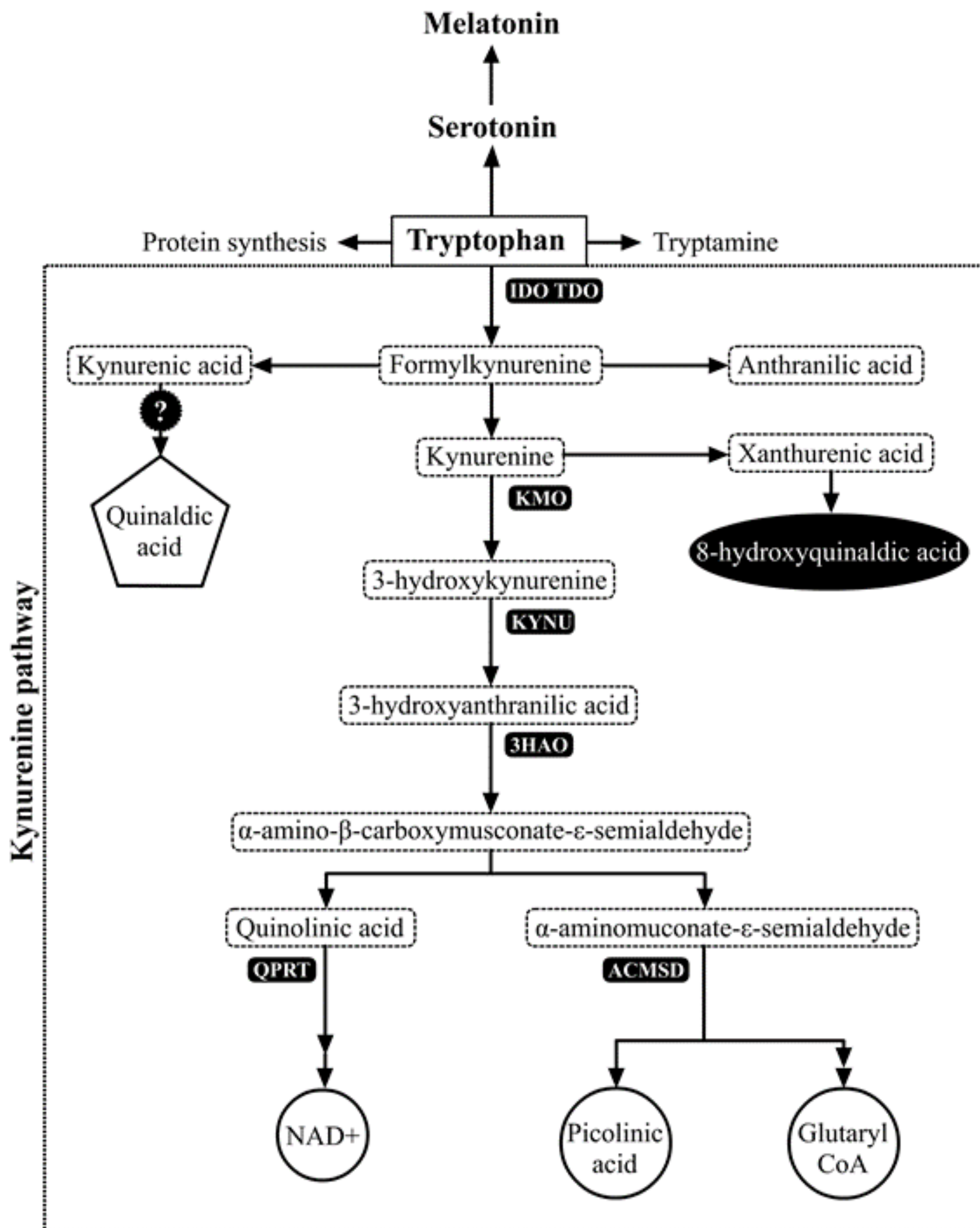


Figure 1. Tryptophan catabolism with particular reference to the kynurenine pathway

2. Monitoring the developing embryo:

The time-lapse technique is an option to monitor the developing embryos for a longer (0-5 day) period. At short intervals photos of the embryo are taken which are then played as a video footage. The main advantage of this technique is that we are able to monitor the development of the embryos without the risk of removing them from the thermostat. With the help of the technique, we are able to detect also the dynamics (apoptosis, necrosis, fragmentation) of embryo development complementing the traditional morphological evaluation. Results are digitalized, archived and can further be compared with any scientific result. Embryos can be cultured individually, or as groups upon request. The thermostat used to culture the embryos is a Binder CB 160 instrument equipped with a Primo Vison EVO microscopic optical unit. Data analysis is carried out using the Primo Vison version 5.0 software. Since the technique is relatively new, we are currently comparing our observations and results with the standard parameters included in the data analysis software.

3. Effects of oxidative stress on fertilization ability:

Oxidative stress has many impacts on human health and on the fertilization ability as well. We developed a chemiluminescence based total antioxidant capacity assay (ECL) and also studied the oxidative stress towards the genetic material by measuring 8-OHDG. Serum and follicle fluid samples were studied during the IVF process of otherwise healthy and of endometriosis patients. We found an inverse relationship between the ECL and the 8-OHDG levels and concluded that the follicle sample was a better predictor regarding the success of the IVF process than serum. A study was performed to evaluate oxidative stress markers of total non-enzymatic antioxidant capacity and 8-hydroxy-2′-deoxyguanosine in the serum and follicular fluid of patients undergoing in vitro fertilization (IVF). Total antioxidant capacity was quantified by enhanced chemiluminescence assay and 8-hydroxy-2′-deoxyguanosine levels was measured by enzyme-linked immunosorbent assay. It was demonstrated that these biomarkers responded to controlled ovarian hyperstimulation differently. Both follicular fluid total antioxidant capacity and follicular fluid -hydroxy-2′-deoxyguanosine had a negative impact on the number of good quality embryos, but an effect of serum TAC and 8-OHdG could not be observed. Women without endometriosis had higher levels of serum and follicular fluid total antioxidant capacity when they progressed to clinical pregnancy. Our findings support that oxidative stress has an important contribution to the reproductive potential in IVF patients. Results were published in Human Fertility.

4. Non-invasive viability assessment of in vitro fertilized human embryos:

We proved the relationship between the presence of haptoglobin alpha-1 subunit in human embryonic medium and the success of in vitro fertilization. After the embryo has been transferred, the remaining culture medium (40 µl) was immediately put to -80 C until the

measurement. An LC-MS method has been developed for the quantification of the haptoglobin alpha-1 fragment. An HPLC coupled micrOTOF accurate mass instrument (Bruker Daltonik, Germany) equipped with an electrospray ionization source (ESI) was used. Mass spectra were collected between 500 m/z and 1500 m/z. Internal mass calibration was performed at the beginning of every run using the peaks of Na+ formate clusters. Patients (mothers) were randomly divided into two investigation groups. Control group: evaluation of the embryos was carried out exclusively according to the traditional, morphological scoring method. Haptoglobin group: the traditional, morphological evaluation of the embryos was supplemented with the results of the mass spectrometric method. Retrospective experiments revealed that the use of the alpha-1 chain of human haptoglobin can be used as a quantitative

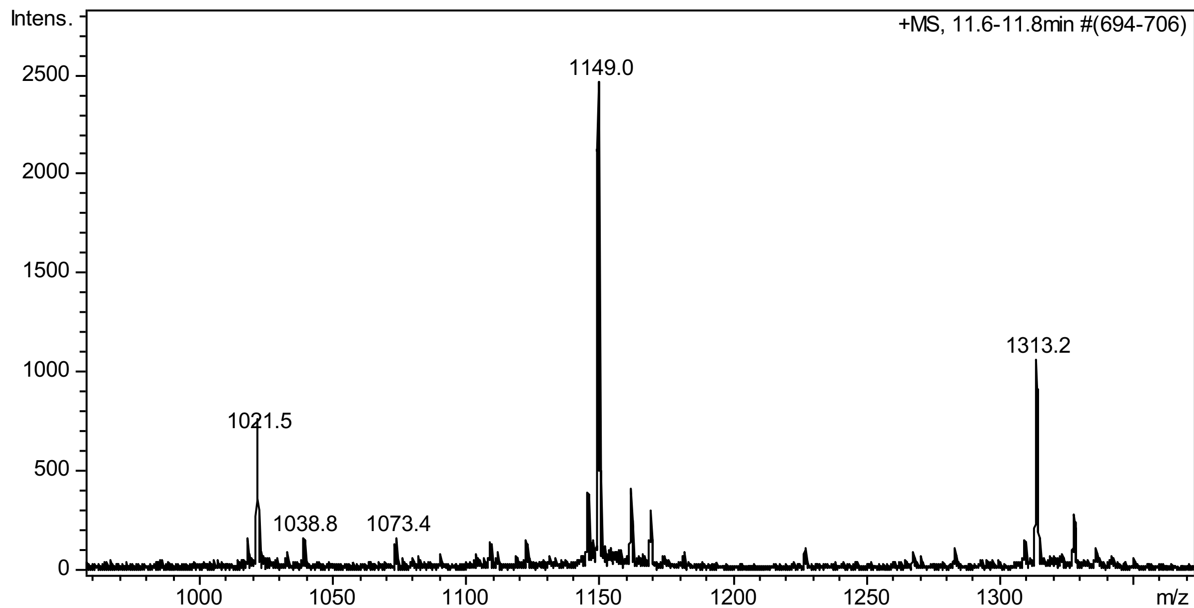


Figure 2. Mass spectrum of the haptoglobin alfa-1 chain. Peaks represent the multiple charged ions of the peptide.

biomarker of viability of in vitro fertilized human embryos. The concentration of haptoglobin alpha-1 was found to be significantly higher in the culture medium samples of embryos representing failed transfers.

The following prospective study contained 100 patients in the “control” group where the embryos were assessed only by morphological evaluation and 100 patients in the “haptoglobin” group where embryos were assessed by both assays. In the “control” patient group embryos graded as “good” or “fair” by the Istanbul consensus morphological grading system were selected for transfer while in the “haptoglobin” patient group embryos graded as “good” or “fair” by the Istanbul consensus morphological grading system and as “positive” by the mass spectrometric assay were transferred. The mass spectrometric assay served as an additional tool to evaluate the order of the selection of the patient’s embryos for transfer. A total number of 1400 culture medium samples were analyzed, concentration values of human haptoglobin alpha-1 were expressed as ng/µl. The average number of embryos

transferred/patient was 2.2, which resulted in a clinical pregnancy rate of 35.2% in the control group while 44.3% in the haptoglobin group. To perform this study, ethical permission was given by the Ministry of Human Resources (10792-7/2017).

5. Correlation of embryo morphology with mass spectrometry:

It was clarified that the amount of haptoglobin alpha-1 fragment used as a quantitative biomarker is truly a new indicator of embryo viability and is independent from the morphological scoring. Comparing the mean fragment amounts in the groups “good”, “fair” and “poor” made by ICCS, ANOVA revealed no significant ($p>0.05$) difference. Correlation analysis between the amount of the fragment or the biochemical diagnosis and the ICCS score “good”, “fair” and “poor” did not reveal any significant correlation (0.094, $p>0.05$). The three described ICCS categories are set by the evaluation of the developing embryos’ morphological aspects. The significance of the difference in the amount of the haptoglobin fragment according to the biochemical diagnosis or the clinical outcome was high ($p<0.001$), the Pearson’s correlation coefficient between the biochemical diagnosis and the clinical outcome was also significant (0.543, $p<0.001$). We observed that the biochemical evaluation can elevate the success rate of IVF by selecting the embryos having good morphological aspects but low implantation potential due to visually (microscopically) unnoticeable reasons. The elevation in the positive prediction is mainly due to the fact that using the biochemical evaluation the number of failed transfers (false positives) decreases.

6. Optimization of the Istanbul consensus viability scoring system:

Two morphological embryo assessment methods were used: the original Istanbul consensus criteria (ICCS) and an optimized criterion system (OCS). It has been found that symmetry of cleavage and mononuclearity in the blastomeres correlated highly with the implantation potential. Symmetric position of blastomeres was classified as good (full symmetry), fair (light asymmetry), or poor (evident asymmetry). A further modification to the ICCS, the assessment of fragmentation was slightly changed. Embryos were considered as good if the fragmentation rate was <15% (instead of the original 10%). When the embryos were evaluated according to the ICCS, pregnancy occurred in almost equal numbers in the good and fair embryo groups. When the embryos were evaluated according to the OCS, pregnancies were assigned to the group of good embryos. Results showed that our optimized scoring was more permissive (good score if the proportion of fragmented cells is less than 15%). We also introduced symmetry and the number of blastomeres, which, were not included in ICCS. Finally, we constructed a composite score based on these parameters. Taken together, the OCS score is more sensitive for the evaluation of Day-3 embryos and gives a composite, more simple and uniform evaluation of both Day-3 and Day-5 embryos.

7. Specificity of disulphide bond reduction within the haptoglobin molecule:

It was revealed whether the disulphide bond reduction within the haptoglobin molecule is an enzyme catalyzed proteolytic process or a non-specific chemical reaction. The mature haptoglobin consists of two disulfide-linked α and β chains. Haptoglobin is fragmented by the cleavage of this disulphide bond. The possible biochemical background can be either the increased release of disulphide bond cleaved by proteolytic enzymes of the non-viable embryos or the alteration of the chemical composition of the surrounding medium resulting in a reducing environment. Organic compounds containing disulphide bonds were incubated in spent culture medium (SCM) samples, and it was examined whether the samples of non-viable embryos reduce the disulphide bonds within these compounds with a higher grade than the samples of viable embryos. Analytical standards of glutathione disulphide (GSSG), glutathione (GSH) and mercapto-succinic acid were used. Mercapto-succinic acid was not available as a disulphide; the solution of the reduced form was subjected to air oxidization of thiol groups. The efficiency of this reaction was detected by direct electrospray ionization (ESI) mass spectrometry. To 25 µl of SCM samples of viable ($n=25$) and non-viable ($n=25$) embryos 5µl of GSSG solution (8 pmol/µl), or solution of mercaptosuccinic acid disulphide (7.5 pmol/µl) was added. The solutions were incubated for three days, under conditions identical to the in vitro fertilized embryos. Samples were measured by liquid chromatography coupled mass spectrometry (LC-MS), cortisol was used as internal standard. LC-MS measurements revealed that there was a significant decrease in the concentration of GSSG ($p=0.001$) or mercapto-succinic acid disulphide ($p<0.001$) in the samples of the non-viable embryo group compared to the control or the viable group. The specificity of the reaction reducing the disulphide bond within the haptoglobin molecule is not only confined to haptoglobin, but probably also to other molecules containing disulphide, as well. The results suggest that haptoglobin fragmentation during early embryonic development is not an enzymatic reaction specific for haptoglobin.

8. Quantitative haptoglobin alpha-1 detection based on lab-on-a-chip technology:

Utilizing antigen-antibody affinity a lab-on-a-chip diagnostic assay has been constructed. In the embryo culture medium multiple forms of haptoglobin are present, however only one, the alpha-1 chain having oxidized cysteine molecules reports information on viability of in vitro fertilized embryos. The performance (specificity, sensitivity, intra- and inter-assay variation) of antibodies was compared between products of common manufacturers. The best result regarding the balance between sensitivity and specificity and best assay-to-assay stability was achieved using monoclonal IgG type antibodies raised in mouse, against epitope containing the “STVPEKKTPKSP” amino acid sequence. Potential antibody combination was constructed though this set up was not specific for the free, soluble form of the haptoglobin alfa-1 chain. Detection performances of fluorophores and chromophores were also compared. It was found that much better parameters of sensitivity were achieved using fluorescent nanoparticles compared to other chromophore labelling. Based on the lab-on-a-chip assay three prototypes

of an integrated point-of-care instrument was also constructed. The instrument is composed of a pre-analytical unit performing the separation of the protein bound and the soluble haptoglobin alfa-1 fraction and an analytical module performing the quantification of the latter. On the TLR scale the innovation represents the TRL-7 level.

9. Effects of fractalkine on implantation and endometrium receptivity:

Fractalkine (FKN) might have an important role in the feto-maternal interaction during gestation since the trophoblast cells express fractalkine receptor (CX3CR1) and the endometrium cells secrete FKN. CX3CR1 controls three major signaling pathways, PLC-PKC pathway, PI3K/AKT/NFκB pathway and Ras-mitogen-activated protein kinases (MAPK) pathways regulating proliferation, growth, migration and apoptosis. We focused on the molecular mechanisms of FKN treatment influencing the expression of implantation-related genes in trophoblast cells (JEG-3 cells) both in mono-and in co-culture models. We established a bilaminar co-culture system in which the trophoblast and endometrium cells (HEC-1A cells) can connect to each other physically. We revealed that FKN increased cell viability through the activation of MAPK pathways via the fractalkine receptor. We determined the levels of progesterone receptor (PR), SRC-1 progesterone co-receptor, activin receptor, matrix metalloproteinase 2 and 9 regulating proliferation, growth, differentiation and invasion in trophoblast cells. In case of HEC-1A cells we examined the expression of activin, follistatin and bone morphogenetic protein 2 (BMP2), which can regulate proliferation, differentiation on invasion of trophoblast cells via the activin receptor and bone morphogenetic protein receptors (BMPRs). Our results revealed that FKN acted in a concentration- and time-dependent manner on JEG-3 cells. The results also suggest that FKN may contribute to successful attachment and implantation of embryo. The study of endometrium receptivity requires a complex network of regulatory factors whom production is strictly controlled especially at the implantation window. We examined the effect of FKN on PR, SOX-17 and NRF2 expressions in HEC-1A endometrial cell line. We found that FKN activated fractalkine receptor signaling and the expression of SOX-17 through progesterone receptor in HEC-1A endometrial cells, and as a consequence it increased endometrial receptivity. Fractalkine also activated the NRF2-Keap1 signal transduction pathway elevating the IL-6 and IL-1β cytokine productions, which increased endometrial receptivity, as well. Based on our results it seems that FKN enhances the establishment of endometrial receptivity.

10. New generation sequencing analysis of cell free medium:

We developed a clinically applicable strategy for non-invasive pre-implantation genetic testing that based on next-generation sequencing. In our experiments we analyzed spent blastocyst culture media of 3rd day embryos fertilized with intracytoplasmatic sperm injection and performing good quality score on morphology assessment. After registered pregnancy outcome spent culture media samples and corresponding blank culture media

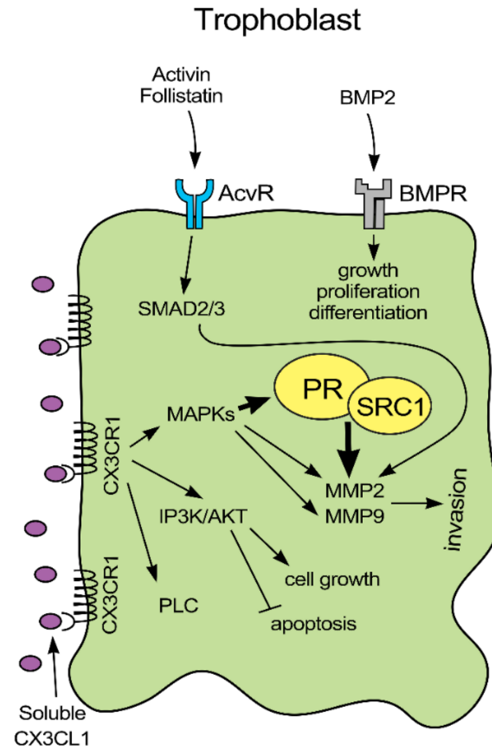


Figure 3. Mechanism of action of fractalkine on JEG-3 trophoblast cells Fractalkine binds to its cognate receptor on the surface of trophoblast cells activating MAPK and AKT pathways. MAPKs can phosphorylate PR on serine amino acid residue increasing its activity. Secreted activin may bind to activin receptor on the surface of trophoblast cells activating SMAD2/3 transcription factors that are also the targets of the BMP2/BMPR signalling pathway. SMAD2/3 transcription factors can upregulate MMP2 and MMP9 expressions. MAPKs are activated by fractalkine signal transduction pathway, act positively on MMP2 and MMP9 transcription. PR influenced by fractalkine can also affect MMP2 synthesis.

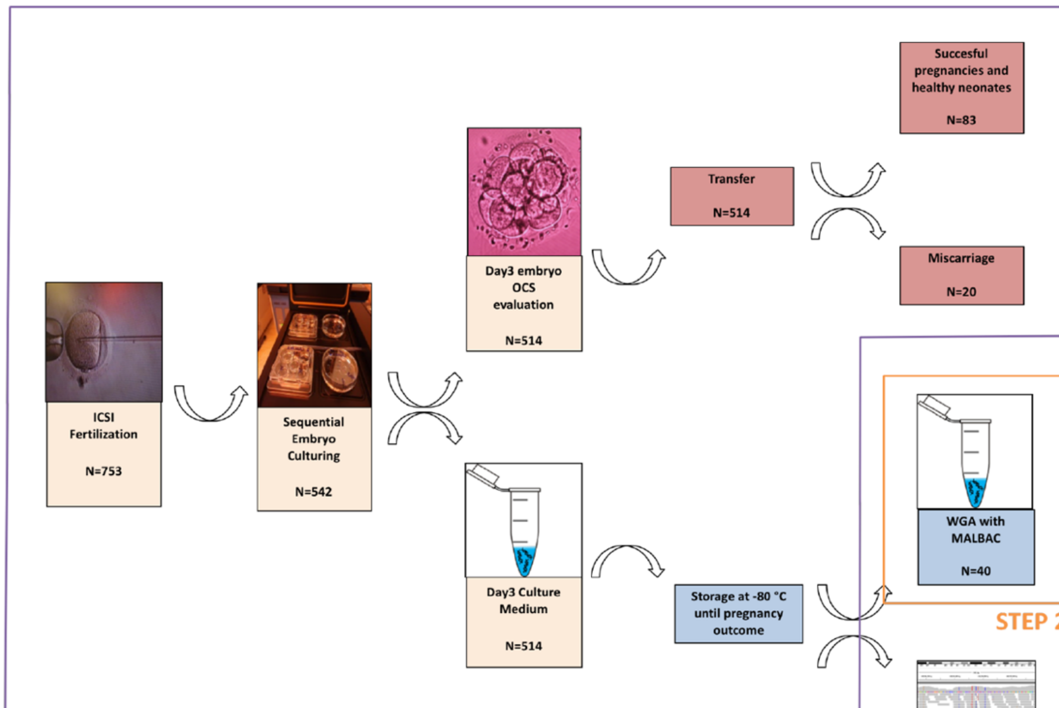
were sequenced on Illumina HiSeq 4000 platform for CNV detection. During the data analyses several CNV detection algorithms were tested to find the best one suit for non-invasive pre-implantation genetic testing method. Our group established an advanced NGS technique combined with downstream application of a bioinformatic workflow which we implied to our study and seem to be an appropriate method to distinguish between contaminating DNA and DNA of embryonic origin even in a very early developmental stage. After identification of the DNA fraction related to the cultured embryo, we found that human embryos that show competence for blastocyst development and successful pregnancy are different in their culture media gDNA content compared with embryos that abort after successful implantation. In particular, analysis of DNA profiles of day 3 spent media demonstrated that higher gDNA copy number is associated with impaired intrauterine development and marked for miscarriage outcome, while low gDNA of embryonic origin in the culture media was found to be characteristic for healthy pregnancy and live birth. We confirmed gDNA content of the embryonic secretome on day 3 to be a putative early biomarker of blastocyst quality correlated to pregnancy outcome.

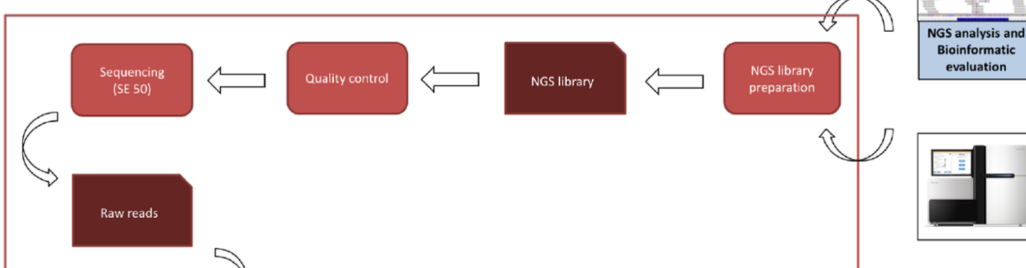
Deeper evaluation of the CNV results in the 1Mb bins of the autosomes and comparing our results with UNIQUE, Genetic Alliance and CDO databases revealed 17 relevant chromosomal alterations. All of these occurred only in the aborted embryo group and was related to registered chromosomal alterations and major developmental impairments. Chromosomal alterations of the embryonic gDNA found in the aborted embryos included 9p12-p11.2: ANKRD20A3 gene inactivation syndromic hydrocephalus due to diffuse hyperplasia of choroid plexus, 11q23.1-23.3: Beckwith-Wiedemann Syndrome, 21q22.3: RIPK4, PCNT popliteal pterygum syndrome, lethal type, also related to Down Syndrome, and 22q13.2-13.31 SCO2 cardioencephalomyopathy due to cytochrome C oxidase deficiency. All the mentioned chromosomal abnormalities are incompatible with life and can be identified with optimized pipeline of NGS analysis non-invasively from blastocyst culture medium at early embryonic stage.

11. miRNA expression patterns of human embryonic culture media:

To assess the role of miRNA associated with embryo competence human embryonic culture media was collected in a prospective cohort study. Seven embryo specific miRNAs (hsa-miR-16, hsa-miR-146a, hsa-miR-191-3p, hsa-miR-302, hsa-miR-372, hsa-miR-221-5p and hsa-miR-486) were selected and analyzed in spent human embryonic culture media of morphologically similar, good quality embryos which after later transfer developed either to healthy embryo and healthy neonate and of those, where missed abortion occurred in early stage of the pregnancy. Spent culture media at the morula-blastocyst stage (3rd day) were collected from embryos fertilized with ICSI and undergoing embryo transfer. After registered pregnancy outcome 10 samples from the cohort of healthy embryos, 9 samples from the missed abortion cohort and their parallel blank culture media samples were enrolled in miRNA analysis.
Isolation and quantitative detection of miRNA from embryonic culture media was carried out on automated droplet digital polymerase chain reaction platform. Quantitative analysis and Kruskal-Wallis statistical evaluation confirmed miR-191-3p ($p<0,001$) and miR-302 ($p=0,02$) to be present in significantly higher concentration in the 3rd day culture media of human embryos than the missed aborted embryos.

All investigated miRNAs were present in the blank media in low amounts except miR-191-3p, which was absent. Focusing on the changes of the background contamination miRNA content, we found miR-221-5p ($p=0,01$) concentrations to be reduced in the culture media samples from embryos with healthy outcomes. In conclusion, expression patterns of miRNA molecules that are present in human blastocyst culture media can reflect the adaptive capacity of the preimplantation blastocysts and non-invasive prediction of implantation success can be assessed.





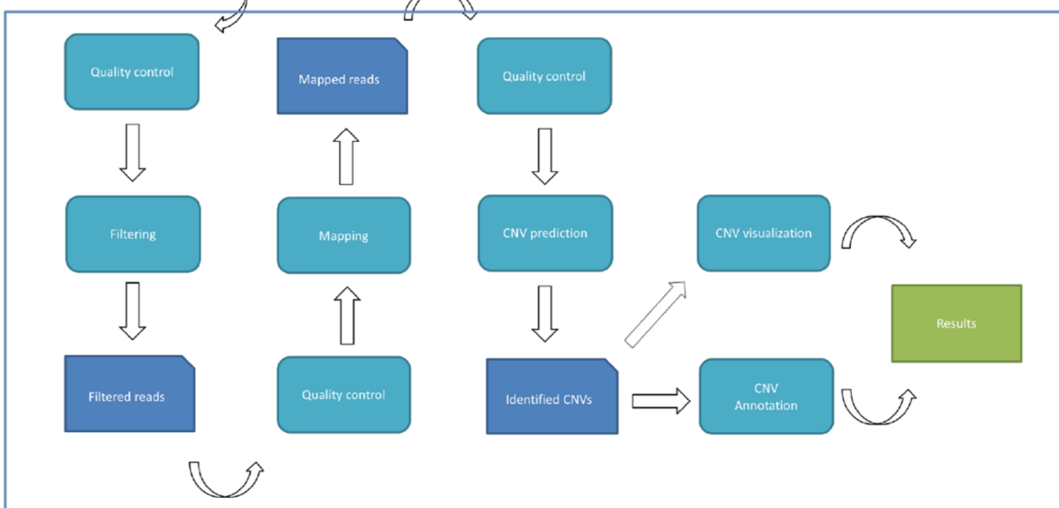


Figure 4. Representation of the entire workflow with all four main steps including Step 1: IVF procedure and sample collection, Step 2: Whole genome amplification. Step 3: Next-generation sequencing and Step 4: Bioinformatics analysis.

12. The effect of light exposure to regulatory miRNA network of the molecular clock and NANOG/SMAD4 mediated cell differentiation

During assisted reproduction the embryos are exposed to stress factors as light exposure. We aimed to investigate the effects of differently administered light exposure to photosensitive genes that maintain circadian rhythm and their molecular regulatory network and the effect on early embryonic development in CD1 mouse embryonic model. In the experimental design in vitro, cultured embryos were exposed to white or red filtered light similarly to embryo manipulation during human IVF procedures. Most of the embryos were then in compacting morula stage. The second light treatment was then performed. The remaining two groups of embryos were exposed to the light treatments under the same conditions. Control embryos were kept in similar culturing conditions as light subjected embryos and during the light exposure they were placed to room temperature at dark room. After embryo harvest from the different experimental groups, we performed droplet digital PCR analysis and immunostaining of the embryos. We found elevated NANOG/SMAD4 expressions especially in in early developmental phase (morula stage) in case of the embryos exposed to white light compared to red light exposed and control, non-exposed, dark cultured embryos. Significantly reduced blastomeric cell number and lesser grade of differentiation was also characteristic for the white light exposed group. These findings correlated with significantly repressed miRNA levels in the white light groups: miR-192 and miR-194 compared to the controls. Molecular clock regulates miR-155 induction that directly repress BMAL and induce PER1. miR-192/194: repress PER 1-3 genes, when levels of regulators are high repression of E-box enhancer element peaks and the molecular circadian clock is reset. In case of miR-192/194 down regulation the circadian cycle is longer that have repressor effect to differentiation processes. In our experiments pre-implantation murine embryos responded to light exposure with altered Per3/clk related miRNA regulation and SMAD4-NANOG expression and impaired embryonic development.

13. The mouse in vitro fertilization model:

The aim of this work was to produce an appropriate number of two cell stage mouse embryos from in vitro fertilized eggs. Methodological steps of the procedure include harvesting of cumulus oocyte complexes (COC) from CD1 female mice. 7-8 weeks old females were treated with 7.5 IU FSH (Folligon), and 7.5 IU luteinizing hormone (Choragon) intraperitoneally. Sixteen hours after the second injection COC-s were gently released from the oviducts into M2 medium (Millipore, England). After washing the COC-s were transferred to the fertilization drop (G-IVF medium Vitrolife, Sweden) and the dish was placed in a 5% CO2 incubator ready for fertilization. Second step was sperm collection from CD1 males. 10-12 weeks old males were sacrificed, the cauda epididymides were removed and placed in a drop of G-IVF medium overlaid with oil. The bleb of sperm was teased out into the medium with a pair of fine forceps

and the dish was placed into the CO2 incubator to allow the sperm to disperse. When the sperm was completely dispersed, 5-20 µl of sperm solution was added to each fertilization drop and incubated for 5 h in CO2 incubator. Fertilization of the oocytes occurs within 1-2 hours. After 5 hours incubation, the denuded one cell stage embryos were washed twice and transferred into the culture (KSOM, Millipore, England) medium. After 26 to 29 h incubation of the zygotes the two cell stage embryos were provided for further genetic investigation. Culture media of the embryos were collected, and freeze stored at -80 grade Celsius till further investigation.

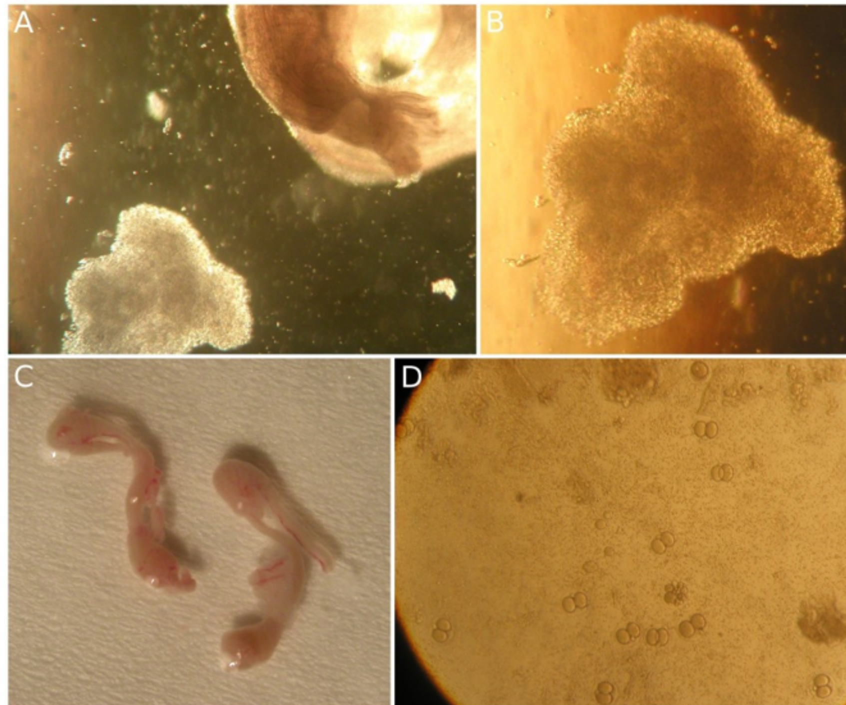


Figure 5. In vitro fertilization in mice. A: Harvest of COC-s. B: COC-s in fertilization droplet. C: Removed epididymes for retrieval of the sperms. D: Embryos on the next day after in vitro fertilization.

Genomic DNA content and cell-free nucleic acid release during early blastocyst development. We used the 2-cell embryonic stage murine blastocyst as a model to compare with the 5-day human blastocysts and their culture media during our experiments for the following reasons: 1. Fertilization triggers the completion of meiosis, followed by the formation of a 1-cell embryo containing haploid paternal and maternal pronuclei before entering the first mitosis to produce a 2-cell embryo containing two diploid nuclei. 2. Transcription from the newly formed zygotic genome, known as zygotic genome activation (ZGA), occurs in two phases: a minor activation before cleavage and a major activation (major ZGA) that is detectable during the 8-cell stage in human blastocyst and 2-cell stage in mouse blastocyst. DNA analysis focused on the detection of apoptosis and mtDNA regulation: PolgA, Gata1, SP1, Fgfr1 and Zfp57 genes. In animal mitochondria, polymerase gamma A (PolgA) is the solitary DNA polymerase. In

contrast to the functional specialization evident in nuclear DNA polymerases, PolgA appears to be uniquely responsible for mitochondrial DNA synthetic reactions in replication and repair. We obtained embryos (n=11) that were fertilized in the same droplet of specific, human serum albumin (HSA)-free culture medium. DNA isolation was carried out using REPLI-G Single-Cell isolation kit (QIAGEN) both on 20ul of the culture media and individually from the blastocysts. In the second set of experiments, we obtained 9 embryos that were fertilized in the same droplet of HSA-containing G-IVF culture media. During the third experiment period, zona pellucida was removed from the blastocysts at two cell stage in an additional 2 cases and embryo-free G-IVF media were collected. The subsequent DNA extraction and linear amplification was carried out. All DNA quantification experiments were carried out on the QX200 Droplet Digital PCR system (BIO-RAD) with EVAGreen detection. Mastermix contained 2xddPCR Supermix for EvaGreen 12ul, custom designed forward and reverse primers for the target genes at 4umol concentration and 100pmol template DNA in each reaction. Embryo-free has containing and has-free media were used as negative controls. We observed different DNA quantities according to the differently collected blastocysts and the corresponding culture medium samples in case of PolgA, Gata1, SP1, Fgfr1 and Zfp57 genes. We could obtain markedly higher gene concentrations from blastocysts hatched from the zona pellucida and their parallel HSA-free G-IVF culture media (Bl-9-10), compared to all other blastocysts with differing collection conditions (BL1-5). DNA concentrations measured in these blastocysts counted up to 136,9 and 148 copies/ul for PolgA gene, with 61,8 copies/ul in the HSA-free G-IVF culture medium, 32,3 and 35,1 copies/ul for Gata1 with 9,3 copies/ul in the culture medium and 0,21 copies/ul for Sp1 with 0,14 copies/ul in the culture medium. Non-hatched blastocysts and corresponding culture media contained 0,06-0,32 copies/ul DNA averagely with a narrow scatter in the results between the different genes. Embryo-free control culture media had the lowest DNA content, we measured only 2 cases out of the 10 when DNA content was other than zero (0,0012 and 0,007 copies/ul). We succeeded to isolate a detectable DNA fraction containing intact DNA fragments from murine embryos removed from the zona pellucida and cultured in HSA-free embryonic media. Highest DNA concentrations were gained for PolgA and Gata1 genes. Embryonic culture media contained detectable fractions of both genes which proportionated nearly half of the concentrations seen in the embryos themselves. (This result might be due to the efficient linear amplification step used in our DNA purification procedure.) Due to the difficulties to remove the zona pellucida in time from the murine embryos in this term we had low number of blastocysts.

14. Development of novel diagnostic methods for diagnosing systemic inflammation in the mother:

Unrevealed systemic inflammation of the mother may be one of the underlying mechanisms for female reproductive disorders. The primary emphasis of our research activity regarding inflammation was to find novel protein inflammatory biomarkers. This effort is of high importance because in the literature more than 200 markers have been described but none

of them proved to be 100% efficient in the clinical practice. We worked out and validated an automated immune turbidimetric test for the measurement of urinary orosomucoid (u-ORM). A dramatic increase of u-ORM was seen in patients with sepsis and u-ORM proved to be an early indicator of sepsis and its level correlated with the severity of the disease. U-ORM was also found to be a promising novel inflammatory marker in patients undergoing cardiac surgery. On the other hand, in severe inflammation intracellular actin is released from the injured tissues causing further complications. We studied the actin scavenger system (actin-gelsolin) in systemic inflammation and verified the decrease of gelsolin parallel to the severity of the disease. First, due to the lack of commercially available reagent kits, this study required western blot method for the quantification gelsolin (GSN) which technique is highly unsuitable for routine laboratory testing. Therefore, we developed and validated an automated immune turbidimetric assay for serum GSN on a routine laboratory instrument. Our automated measurement further verified the diagnostic value of serum GSN determinations in systemic inflammation.

Autoimmune diseases quite often have a negative impact on fertility. Urinary orosomucoid (u-ORM) is a novel marker of systemic inflammation. We studied u-ORM referred to creatinine (u-ORM/u-CREAT) in adults and in children with active and non-active Crohn's disease (CD). We found seven times higher values in children with active CD (0.50 vs 0.07 mg/mmol, $P < 0.001$) and two times higher in adults (0.32 vs 0.14 mg/mmol, $P = 0.01$) compared to patients with inactive disease. U-ORM/u-CREAT showed good correlation with conventional inflammatory markers (hs-CRP, serum ORM; $P < 0.01$) and activity indices (HBI, $P = 0.018$; PCDAI, $P < 0.001$). U-ORM/u-CREAT had similar discriminative performance to hs-CRP and serum ORM in the differentiation of active from inactive pediatric CD patients.

For the laboratory diagnosis of systemic bacterial inflammation and the evaluation of the disease severity novel biomarkers were used. Besides the adaptation of the immune turbidimetric assay of serum gelsolin (GSN) we also worked out and validated a Gc globulin assay (both two are actin binding proteins) to automated determination in our cobas 502 analyzer (Roche Diagnostics). Data revealed that first-day GSN differentiated sepsis from non-sepsis (AUC: 0.88) similarly to C-reactive protein (AUC: 0.80) but was slightly inferior to procalcitonin (PCT) (AUC: 0.98) with a cutoff value of GSN at 22.29 mg/L (sensitivity: 83.3%; specificity: 86.2%). Only first-day SOFA scores (0.88) and GSN (0.71) distinguished septic survivors from non-survivors, whereas lactate (0.99), Gc globulin (0.76) and mean arterial pressure (MAP) (0.74) discriminated septic shock from sepsis. Logistic regression analyses revealed SOFA scores and GSN being significant factors regarding 14-day mortality. First-day GSN levels were higher ($p<0.05$) in septic survivors than in non-survivors. Gc globulin levels remained higher ($p<0.01$) in sepsis when compared with septic shock during the follow-up period.

Further diagnostic biomarkers in systemic inflammation: besides the previously developed automated laboratory methods for the rapid determination of GSN, Gc globulin and u-ORM, the measurement of a novel urinary biomarker was necessary that can predict acute kidney failure in systemic inflammation. Severe inflammation can cause acute tubular kidney injury

which precedes glomerular failure, but which is difficult to diagnose. Serum Cystatin C (CysC) is a well-known marker of glomerular function however, it can also be found in the urine in very small amounts (u-CysC). U-CysC levels reflect tubular functions therefore, an increase in u-CysC might be a good early indicator of tubular damage. Unfortunately, there is no commercial diagnostic kit for u-CysC determination. In our work, we adapted and fully validated a serum CysC reagent-based method for automated urinary CysC measurement (measurement time 10 min). Our clinical evaluation strongly supported the usefulness of u-CysC as an early biomarker of acute kidney tubular injury.

Tissue culture models are also of great importance in testing different compounds that affect viability of the cells (toxins, metabolic inhibitors, activators of inflammatory processes, etc.). It is of utmost interest to have precise information on the well-being of the cells, especially that of the embryos (in animal experiments). Therefore, we worked out various viability assays. First, we developed a multiparametric method utilizing a cell line (A549 lung cancer culture) that expresses green fluorescent protein (GFP) at a constant rate. In our study the cells were treated with metabolic inhibitors and were lysed and stained by propidium iodide. Our novel microplate method was suitable for the parallel measurement of nucleic acids, GFP, ATP and protein levels. ATP and GFP were referred to total protein and total nucleic acid content from the same sample. The data were further verified by flow cytometry, including measurement of apoptosis/necrosis rate. By comparing the data obtained from the microplate and the flow cytometry assays we could have information on the mode of action of the treating compounds. Our cellular models are of great importance in studying the effects of various protein/non-protein compounds affecting cell viability.

In liver cell cultures (HepG2) a novel enzymatic fluorescence intracellular glucose microplate assay was developed. Our method was suitable for simultaneous determination of intracellular ATP and protein measurements as well. We could detect the cellular effects on cell metabolism using 10 different metabolic and/or glucose transporter inhibitors. We also studied the glucose uptake of the treated cells by flow cytometry using 2-NBDG fluorescent glucose analog.

For single cell viability assay the 2-NBDG fluorescent glucose analog could also be used to quantify the glucose uptake by fluorescence microscopy and digitalization of the fluorescence signal inside the cells.

Finally, as a non-invasive method to predict inflammatory processes we developed a fluorescence Elisa assay for the quantitative determination of urinary Gc globulin. The technique is based on competition between the microplate-bound Gc globulin and standards/controls/samples using rabbit polyclonal anti-Gc globulin IgG and a peroxidase labeled anti-rabbit IgG (secondary antibody). The detection of the formed complexes is done by filling the wells with phosphate buffered H_2O_2 and Ampliflu Red fluorogenic molecules. After 30 min incubation the fluorescence is measured by a plate reader. The method is almost fully validated. Analyzing urine samples from healthy individuals and from patients with systemic inflammation we found a significantly increased urinary Gc globulin in the patient

group. Our novel biomarker analyses might be used as complementary diagnostic approaches for the deciphering of inflammatory processes in the human body.

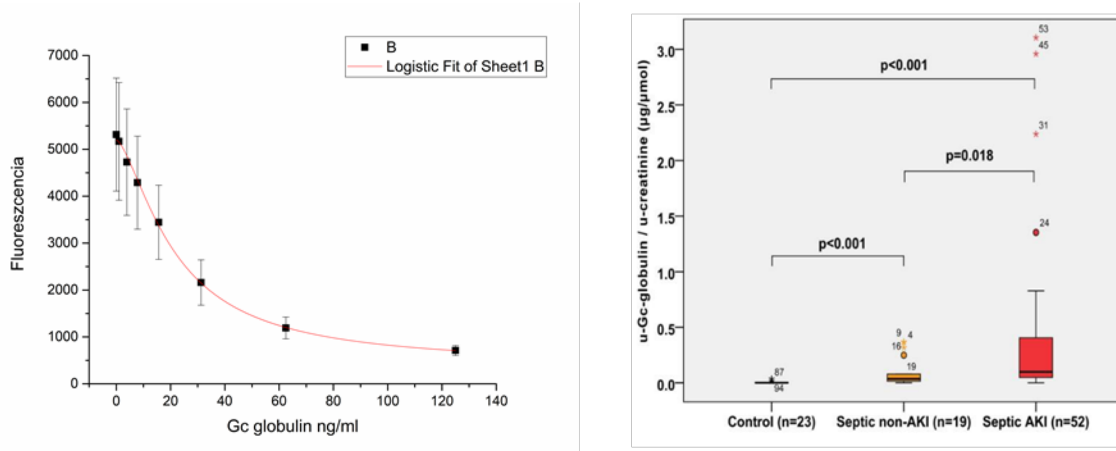


Figure 6. Cumulative calibration curves (n=12) of the Gc globulin fluorescence Elisa method (a) and statistical evaluation of the data obtained from urine samples of healthy controls, septic patients without, and with acute kidney injury (AKI) (b).

15. In conclusion, human reproduction is very inefficient and the number of infertile couples is high. ART have facilitated the birth of over 8 million children worldwide. ART, however, superimposes its own inefficiency upon the inefficiency of normal reproduction. Transferring multiple embryos in an ART cycle has led to an „epidemic" of multiple pregnancies. These considerations point to the great importance to identify biomarkers during embryogenesis, which can predict a normal neonate. In the present project we aimed to improve the success rate of implantation by optimizing morphological grading and studying its correlation with some proteins (e.g., a haptoglobin fragment) in the spent embryo culture medium. Various substances of embryonic origin (cell free DNA, regulatory microRNAs, energy-regulating mitochondrial DNAs, fraktalkin) had been analyzed from the spent embryo culture medium with a wide range of cutting-edge molecular methods, including next generation sequencing. The ultimate aim of the proposal was to find the most viable, healthy embryo for maternal transfer, by understanding the "message" of biological signals sent by the embryo to its fluid environment during the first days of ART.