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Nimet ERGIN

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Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollten dennoch eine Urheberrechtverletzung bekannt werden, bitte ich um Meldung bei mir.

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I. INTRODUCTION

1. Apoptosis

Carl Vogt was the first to describe this very important phenomenon in 1842, but in 1972, John Kerry induced the name of Apoptosis, which is Greek meaning “falling down”. During the past few years there had been many projects concerning apoptosis, because of its importance in several diseases, like cancer, where insufficient apoptosis is occurring, or AIDS and Parkinson, where there is too much (Lawen 2003).

Apoptosis, also called programmed cell death, is a mechanism of the body, in which the cell induces its own death to retain the cell population in balance. If that does not function in a normal, regular way, then the tissue shrinks or grows in a pathological way (Chaffey 2002). Apoptosis is stopped through various physiological or pathological stimuli. The two main parts of the apoptotic mechanism are, the condensation of the nucleus and the breakdown of the cell in separate pieces (Kerr 1972). Next the affected cells fall in small “apoptotic bodies” (Figure 1). The neighboring cells and macrophages recognize these small bodies and phagocyte them. This way of apoptosis, in contradiction to necrosis, does not cause inflammation, because the cells do not lose their cellular constitutions. (Elmore 2007). Cell death is a response of cell proliferation. Physiological apoptosis is a very important and essential process for a healthy metabolism. Two pathways induce apoptosis, the intrinsic one in mitochondria and the extrinsic pathway using cell surface receptors (Crow et al. 2004) . Apoptosis, as already stated, can be activated through two different pathways, either intrinsic (mitochondrial pathway) or extrinsic (death receptor pathway). The regulators of the extrinsic apoptotic pathway are tumor necrosis factor receptors (=death receptors), on which ligands, like FAS and TNFα, can bind (Melino et al. 2006).

The pathway begins with the binding of the ligands FAS ligand or tumor-necrosis-factor alpha (TNFα). (Taylor et al. 2008). An intracellular death domain of the death receptors is crucial for transmitting the death signal. FAS ligand binding to FAS-receptor causes homo-dimerization of the receptor thus the activated receptor binds Fas-associated death domain protein (FADD) and procaspase 8 to form a death-inducing signaling complex (DISC). DISC leads to auto activation of caspase-8 (Beurel et al. 2006). TNF-α binds to TNF-α receptor, and after dimerization the active receptor binds to TNF receptor type 1-associated death domain protein (TRADD), followed by FADD recruitment and also in this way of extrinsic apoptosis the DISC is formed and auto activates caspase-8 (Figure 2) (Elmore, 2007). Caspase 8 activates caspase 3, leading to apoptosis.

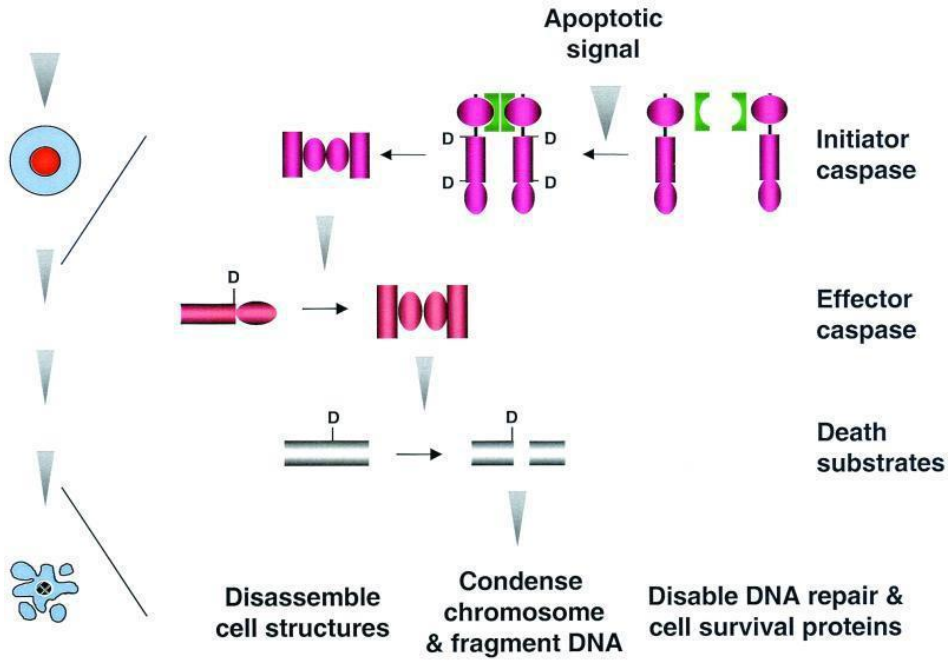

Figure 1:.Activation of caspase cascade (Chang and Yang 2000).

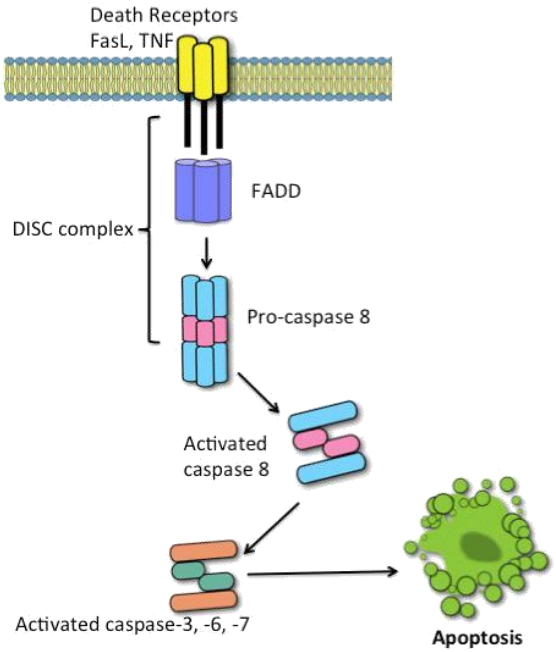

Figure 2 : Activation of extrinsic pathway of apoptosis after lingand binding (Cooper 2012).

The Bcl-2 family proteins regulate the intrinsic apoptotic pathway. There exist pro- apoptotic and anti-apoptotic Bcl2 (B-cell-lymphoma 2) proteins. A membrane anchor, at C-terminus side, of a specific domain including BH1, BH2, BH3 and BH4, divides the antiapoptotic group (for example Bcl-xL). (Greenhalf 1996). Pro apoptotic Bcl2 proteins are divided into two groups, the multidomain pro-apoptotic (Bax and Bak) and the BH3 only pro apoptotic (Bid, Bad, Puma and Noxa) (Zhang et al. 2013; Geng et al. 2010). Triggers like oxidative stress, DNA damage, growth factor withdrawal and therapy with toxicological drugs, activate the mitochondrial pathway. After the signal, mitochondria let cytochrome c free, which binds with APAF-1(=Apoptotic Protease Activating Factor 1) in the cytosol. (Lawen 2003).

The next step is the activation of the caspase 9 (=cysteine aspartic proteases) through the apoptosome (combination of Procaspase9 and Afap1). Caspase 9 activates caspase 3, which is an effector protease and causes apoptosis (Bratton et al. 2010) (Figure 3).

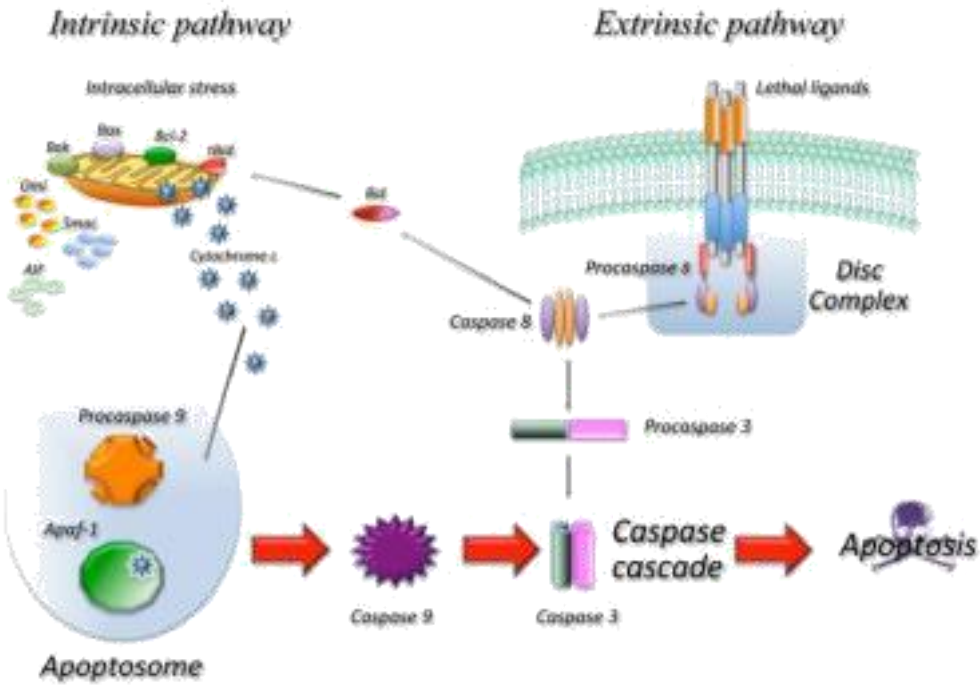

Figure 3.Intrinsic vs. Extrinsic apoptotic pathway (Favaloro et al. 2012).

2. p53

Products of Genes Transcriptionally Activated by p53

p21, WAF1: Function as an inhibitor of several cyclin-dependent kinases.

MDM2: Is a oncogene product and inactivates p53-mediated transcription, that shapes an auto regulatory loop with the activation of p53.

GADD45: Is necessary for induction upon DNA damage; after binding to PCNA the cell cycle can be arrested. This gene is involved in DNA nucleotide excision repair (=NER).

BAX: A BCl2 family member which is involved in promoting apoptosis.

IGF-BP3: The insulin-like growth factor binding protein-3; blocks the signals of a mitogen growth factor.

(Levine et al. 2009)

In 1979, p53 was discovered and 10 years later was characterized as a tumor suppressor gene (Kruse et al. 2009; DeLeo et al. 1979)

P53, is also called “the guardian of the genome”, is a transcription factor which acts in an ant proliferative way and consequently exerts tumor suppressor function.

DNA damage and hypoxia are reasons for p53 activation (Zilfoul et al. 2001). Functions of p53 consist of transcriptional activation, DNA repair, mitochondrial membrane permeabilization (apoptosis), senescence and cell cycle arrest. (Vousden et al. 2005).

After certain stimuli, the “antirepressed” p53 has to be, stabilized and activated, through post-translational modification so that p53 becomes free from MDM2 (=mouse double minute 2)(Lavin et al. 2006). Mdm2, being the opponent of p53, inhibits the stabilization and activation, because it binds on the N-terminus domain and acts against the transcriptional

process. Thus MDM2 is an oncogene (Oliner et al. 2003). Through phosphorylation the binding of MDM2 is inhibited (Zilfoul et al.2001).

After activation and stabilization, p53 binds to DNA via N-terminus. P53 is a 393-amino-acid protein with a 191-amino-acid protease resistant fragment, which consists of the DNA binding domain and the 53-amino-acid carboxyl terminal domain (Pavletich et al. 1993). As a transcription factor, p53 controls the gene-expression which influence cell-cycle arrest, DNA repair, cellular function and also apoptosis. After the binding process, genes of p53 will be activated or repressed, only by interacting with TAFs/TFIIDs (=transcriptional factors). The posttranslational modification of p53 determines which promoter will bind with whom (Zilfou et al. 2001)

2.1. mitochondrial p53

One of the tasks of p53 is apoptosis, after signaling to certain proteins like BAX, CD 95, PIG3 BH3-only proteins like PUMA (=p53 upregulated modulation activator of apoptosis) and NOXA.

P53 interacts with several of the anti- and proapoptotic members of the Bcl2 family. The guardian of the genome reacts with Bcl-xl and Bcl 2 to avoid the inhibitory effect on Bak and Bax, which are proapoptotic proteins. It also blocks the interaction of tBid with Bcl-xL and Bcl-2. In cytosol, the impact of PUMA p53 with Bcl-xL can directly influence Bax.

All these processes induce an effect, called oligomerization and form a pore in the mitochondrial membrane (Figure 4, Vaseva et al. 2009). Researches have also shown, that PUMA has a special influence on the mitochondrial apoptotic pathway, because the loss of PUMA leads to change the activity of p53 concerning to its mitochondrial apoptotic pathway. PUMA is the only target gene which possess such an ability (Jeffers et al. 2003).

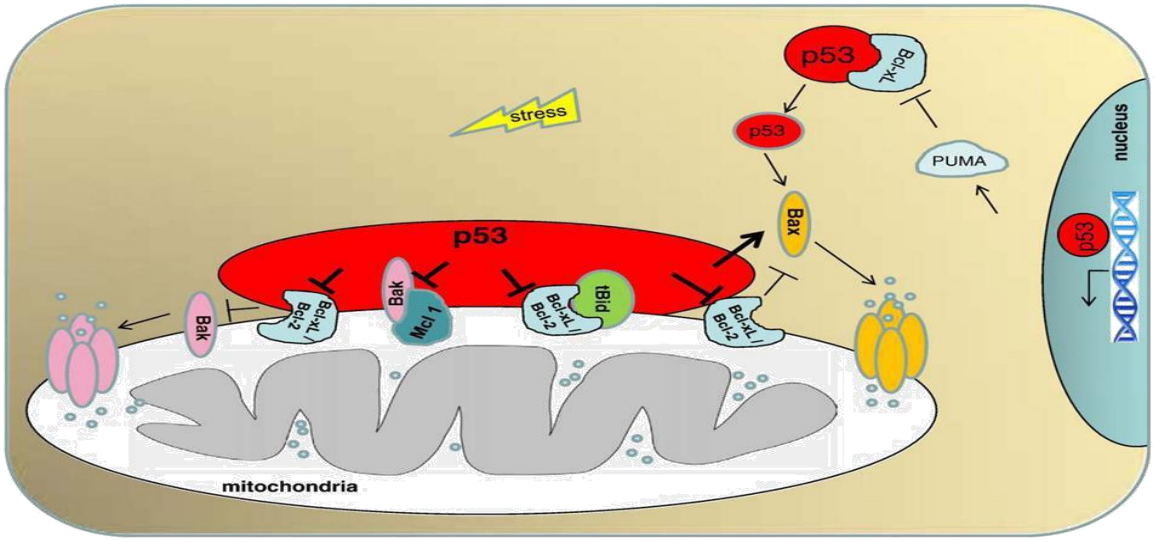

Figure 4: Mitochondrial apoptotic pathway of p53. (Vaseva et al.2009) "The Mitochondrial p53 Pathway." *Biochemical et biophysical acta)*

Besides its function as a transcription factor and mitochondrial apoptosis inducer, p53 includes a cyclical checkpoint during cell division.

G1 cell cycle arrest occurs after the induction of p21WAF1/CIP1/SDI1, which is a cyclin depended kinase inhibitor. Exogenous signals lead to activation of 14-3-3-sigma, which accordingly results in G2 checkpoint arrest (Hermeking et al. 1997).

Studies showed that p53 is also able to interact with the target gene, DRAM (=damage regulated autophagy modulator) which codes itself into a lysosomal protein and leads to macro autophagy. So that is the process of p53 mediated cell death (Crighton et al. 2006).

2.2. DNA-repair mechanism of p53

The guardian of the genome plays also a role as a repair mechanism for DNA. P53 binds onto single strand DNA with two genes, *p48DDB2* and xeroderma pigmentosum complementation

group C (*XPC*). After its interaction with the DNA, the repair mechanism, nucleotide excisions repair (NER), commences its operation. (Smith et al. 2005). A study evidenced that a loss of p53 or gadd45, which is a downstream effector gene of the p53 pathway, can in turn influence global genomic repair (GGR). GGR is a sub pathway of NER (Smith et al. 2000**).**

To promote NER, p53 has to bind to NER repair factors, XPB and XPD, but the tumor suppressor gene is also able to get directly in contact with the exact damaged DNA part. After a DNA damage, cells have the chance to repair themselves during the cell cycle arrest. This is a mechanism to avoid developing cancer because of a mutation, which can be caused by ionizing radiation, ultraviolet light and oxidative stress (Chatterjee et al. 2005).

In a recent study there was clearly shown that a decreased activity of p53, influences 8-oxoguanine DNA glycosylase (=OGG1) activity in a negatively way (Chatterjee et al. 2005). OGG1 encodes the enzyme which is accountable for a mutagenic base byproduct the excision of 8-oxoguanine, a result of exposure to reactive oxygen. OGG1 includes lyase activity for chain cleavage. In 8-OH-dG two groups exist: OGG1-α and OGG1-2a, 2b, 2c, 2d, 2e (Type2). Research has shown that Type 1 changes similarly to 8-oxo2dG. 8-oxo2dG is a marker of oxidative DNA modification, thus the more 8-oxo2dG is produced, the more OGG1 is expressed to repair this damage (Dezor et al. 2011).

One of the promotors of such an oxidative DNA damage is inflammation (Morreall et al. 2014). A repair mechanism, such as BER, (base extinction repair), is produced by glycosylates, which hydrolytically, divide the base-deoxyribose glycosylic bond, of the damaged part of DNA (Lindahl et al. 1999, Sokhansanj et al. 2002). Studies showed that p53 interacts with BER by stimulating this repair mechanism (Chatterjee et al. 2005). OGG1 is one of the most important downstream targets of p53. In another study (Zhan et al. 1993), p53 activity was determined by its capacity to transactivate gene promoters with the help of p53-

binding site. The region between -945 and -726 of OGG1 promotor interacts with p53 regulatory elements, that means an influence on p53 will also change OGG1 activation (Chatterjee et al. 2005).

3. Sepsis/ICU/Apoptosis

Despite many improvements, sepsis is a main death cause of intensive care unit patient. Concerning medical equipment and antibiotic medication, the rate of sepsis cannot be decreased. The incidence of sepsis increases 1,5% per year (Angus et al. 2000). Primarily the sepsis causing apoptosis plays a very important part to the condition of the ICU patients. It is shown that sepsis leads to apoptosis of the immune cells (Ayala et al.1996). Normally the immune response acts with pro and anti-inflammatory factors in balance, but in the case of sepsis a dysfunction exists (Dellinger 2003). The first pattern of the immunological response of sepsis is the overproduction and distribution of pro inflammatory cytokines, like IL-1 and TNF-α, the so called "hyperinflamation". The following process is the "hypoinflamation", which is caused because of too little response of immune cells (Lang et al. 2009). Cytokines lead to fever, anorexia, activation of coagulation cascade, sleepiness and increase in the vascular permeability, which lead to hypotension (Sommers 2003). This imbalance of the organism can cause immune suppression, tissue damage, organ failure and consequently death (Bone et. al. 1992). Sepsis is divided into three types: septic inflammatory response syndrome (SIRS), severe sepsis and septic shock. Severe sepsis is defined, when the above symptoms are combined with one or more organ dysfunctions. The septic shock is a severe sepsis with hypotension, defining an acute situation of the organism (Remick, 2007). SIRS is a systemic inflammation reaction without an infection and typical symptoms are, body temperature under 36°C or over 38°C, pulse upper than 90, a need of mechanical ventilation because of respiratory problems and the white blood cells
are also affected, namely more than 12,000/µL (=leukocytosis) or fewer than 4,000/µL white blood cells count (=leukopenia) or more than 10% immature bands (Bone et. al. 1992). Many ICU patients develop SIRS without any evidence of microbial pathogens in their organism.

After tissue damage the body may release mitochondrial DNA that had a similar structure to bacterial DNA. The mitochondrial DNA bind to toll-like receptor 9 on immune cell and activates the innate immune system. A study tested 228 consecutive patients admitted to a medical ICU between August 2012 and August 2013. The aim of the study was to get the association of mitochondrial DNA levels with 30-day survival and to analyze whether the predictive value is modified by the expression of its receptor toll-like receptor-9. At admission the mitochondrial DNA value was significantly higher in non-survivor in comparison to survivors and patients who had plasma levels at the highest quantile had a 2.6-fold higher risk to die. The study also showed that patients with higher expression of toll-like receptor-9 demonstrate a higher risk associated with increased mitochondrial DNA value when compared with low toll like receptor-9 expression (Krychtiuk et al. 2015). This suggests that the high circulating levels of mitochondrial DNA within increased toll-like receptor-9 expression could be the leading cause of SIRS.

4. Aim section

ICU patients are of main importance. Because of their critical health state, every mechanism that delivers a worse condition, has to be pointed out and even prevented. We aimed to search whether apoptosis plays a different part in ICU patient`s organism.

The primary objective of this task, was to evidence the increased apoptosis of mononuclear cells, in critically ill patients, in comparison to stable patients and to make an assessment about apoptosis induction of ICU serum treatment on PBMC, when compared with healthy human serum pretreated mononuclear cells.

In the diploma thesis other main points were to demonstrate the relationship between p53 value and apoptosis, which p53 downstream targets, like BAX, FAS, GADD and OGG1 are activated and the dependence between p53 and the targets.

The p53 inhibitor, Pifithrin α, was used to block p53 and thus to reflect the cell activity connected to apoptosis.

II. Materials and Methods

Isolation of PBMC (Peripheral Blood Mononuclear Cell)

For PBMC isolation we used LeukosepTM, which is a 30 milliliter centrifuge tube with an integrated porous barrier made of transparent polypropylene. Leukosep is a simple way of separating PBMCs from the unwanted part of the blood like erythrocytes and granulocytes. At first, the tube was filled with 15 ml separation medium and centrifugated for 30 seconds at 1000xg and RT. After centrifugation the separation medium is located under the porous barrier. The next step was to pour the whole anticoagulated blood directly into the Leukosep-tube. Next, we centrifugated 10 minutes at 1000xg and RT without break. After centrifugation, on the base layer were the pellets of erythrocytes and granulocytes, next layer was the separation medium, and over the medium was the porous barrier. Over the barrier was a low amount of separation medium and at the next layer were the wanted PBMCs with plasma.

The supernatant of the content was poured from the Leukosep tube into another centrifugation tube and was washed twice with PBS. (Instruction Manual, Greiner Bio-one)

Stimulation of the PBMCs with ICU (=intensive care unit) serum

Serum of patients from the ICU unit was obtained using serum separation tubes. PBMC were seeded in a density of 1 million cells per 1 ml in M199 containing pen strep, fungizone and glutamine and stimulated with either 10% ICU serum or 10% serum from healthy control subjects.

Staining of cells with Annexin V to determine apoptosis (extracellular staining):

After incubation for 48h, the cells were transferred in Eppendorf for staining

The cells were washed twice with cold cell staining buffer, and were resuspended in Annexin V binding buffer (eBioscience) at a concentration of 0,25-1.0 x 10^7 cells/ml.

100 microliter of cell suspension was transferred in a 5 ml test tube.

To stain phosphatide serine residues we added 5 microliter of FITC conjugated Annexin V (eBioscience) antibody.

Next add 5 microliter PI (eBioscience) viability staining solution.

After gently vortexing, the cells were incubated for 15 minutes at room temperature (25°C) in the dark.

400 microliter of Annexin V binding buffer was poured in each tube and the cells were ready to be analyzed with flow cytometry.

Staining of cells with antigens for flow cytometry (intracellular staining)

After two wash steps the cells were adjusted by adding another 100 microliter of IC fixation buffer (eBioscience), while vortexing the tube.

Then an incubation for 20 minutes in the dark at room temperature.

Without washing, 1ml of permeabilization buffer (eBioscience) were added.

The sample was centrifugated at room temperature for 5 minutes, and then the supernatant had to be discarded.

Cell pellets were resuspended in 1 ml of a permeabilization buffer (eBioscience).

The sample was again centrifugated at room temperature for 5 minutes and the supernatant was again discarded.

With 1 ml permeablization buffer the cells got resuspended again. The next step was centrifugation again.

100 microliter permmeabilization buffer came into each tube and the recommended amount of fluorochrome-labeled antibody (Santa Cruz) for detection of intracellular antigen to cells was added for 20 minutes. Two more repetitions of the wash steps were needed, and the ready cells were resuspended in an appropriate (approximately 400 microliter) volume of flow cytometry staining buffer.

Staining unconjugated primary antibodies and fluorochrome - conjugated secondary antibodies (Santa Cruz)

- Unconjugated primary antibodies to tubes were added (approximately 1 µg per tube).
- 100 µl of the prepared cell suspension were added to each tube.
- After vortexing the cells were incubated for 15-30 min.
- 1 ml of PBS got into each tube.
- Then cells were centrifuged for 5 minutes at 2000 RPM.
- The supernatant was aspirated.
- 100 µl PBS were added to each tube. Add fluorochrome-conjugated secondary antibodies (for OGG, GADD) to tubes. Use 0.5-1 µg of antibody.

- After vortexing 15-20 minutes incubation time had to be hold.
- The cells were washed with PBS and centrifuged for 5 minutes at 2000 RPM. Next the supernatant was aspirated.

  The ready cells were resuspended in an appropriate (approximately 400 microliter) volume of flow cytometry staining buffer

Fluorescence assisted cell sorting (FACS)

The FACS method, a flow cytometry, for dividing a cell population into individual cells. The scattered light and fluorescence capacity makes this process possible (Davies 2007). The process begins with the vibration of a nozzle, in a periodic interval, so as to get a drop. A laser scans the cell assisted fluid during the fall (BD Bioscience). Antibodies which are linked to a fluorescent dye, can be used to sort cells into a certain subpopulation. The chosen antibody possess a binding side to the protein of the specific cell (BD Bioscience, Introducing to flow cytometry). Every antibody bound cell can be detected by FACS laser beam and in addition proteins can be analyzed by metering the main fluorescent intensity (MFI) (BD Bioscience) (Figure 5).

In data analysis, the system of FACS uses two different types of representing the results: dot-plot and histogram (Figure 6,7). The histogram measures one parameter, in contrast to the dot-plot which compares two or three parameters, in two or three dimensional plots (BD Bioscience)

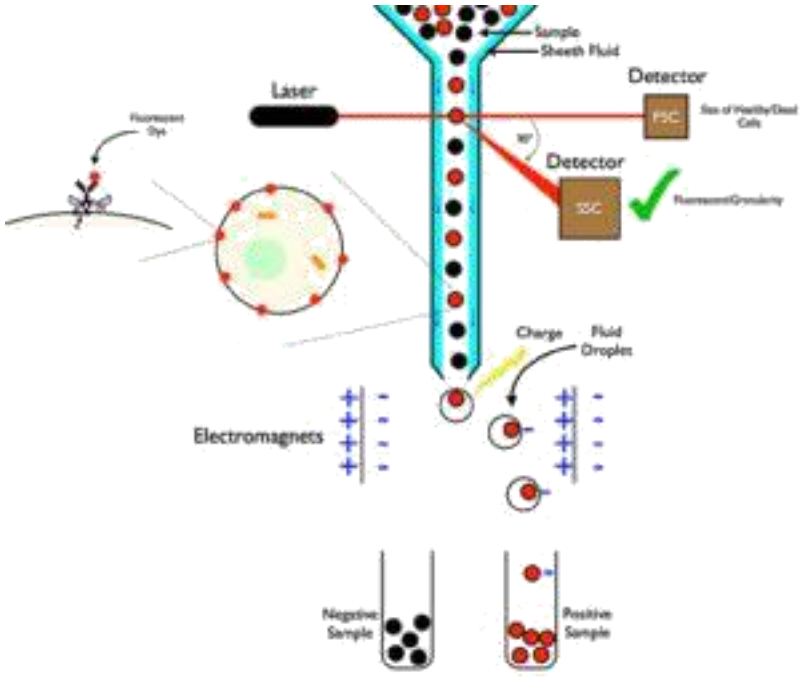

Figure 5: The principle of Fluorescence assisted cell sorting. (Sabban 2011)

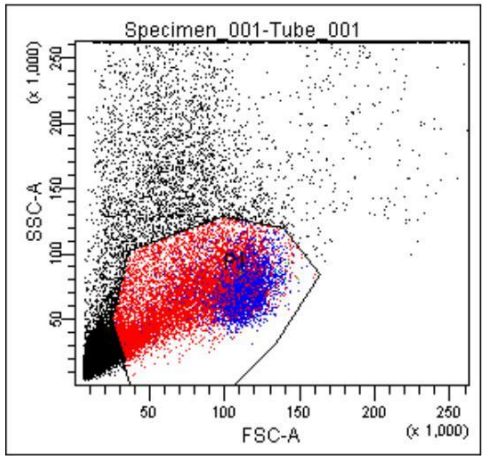

Figure 6: Dot-plot

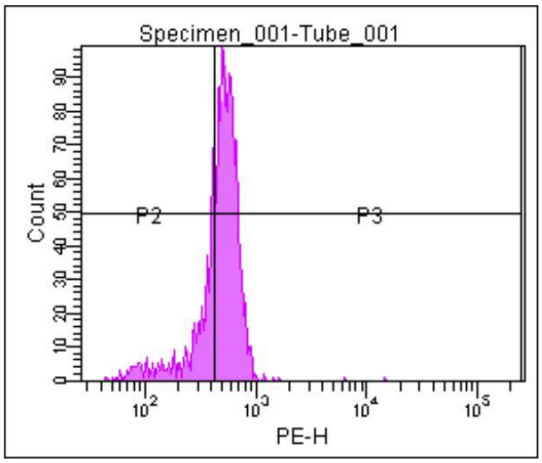

Figure 7: Histogram

RNA isolation from whole blood from ICU patients (Roche Life Science)

400µl lysis/binding buffer is added to 200µl of whole blood and vortexed for 15 seconds. The sample is brought into the upper reservoir of a high pure filter tube, which has to be inserted

to a collection tube. After the sample spins for five minutes at the speed of 8000 rpms, the flowthrough is discarded and the same filter tube is connected to a collection tube. 90µl DNase I incubation buffer are mixed with 10µl DNase I, respectively, in a sterile reaction tube and this solution is inserted to the upper reservoir, of the high pure filter tube and incubated for 15 minutes in room temperature. Next 500µl wash buffer was added and centrifugated under the exact same conditions. After two wash steps (first with 500µl, then 200µl) with wash buffer II, the filter tube is divided from the collection tube without any contact with the flow through. For the last wash step it is spinned for 2 minutes at maximum speed (13000 rpm) to remove the wash buffer.

The filter tube is inserted in a sterile 1,5 mircocentrifugate tube. 100µl elution buffer is added to the upper reservoir to elute the RNA. In the end, after centrifugation for 1 minute at 8000 rpm, the microcentrifuge tube contains the purified eluted RNA. (RNA Preparation-Roche).

Reverse Transcription into cDNA

The reverse transcription is necessary to perform PCR on RNA samples. At the start a complentary DNA copy (cDNA), which is a double-strand, of the single stranded RNA with the help of reverse transcriptase (RT) is created. An oligonucleotide primer binds to the RNA and leads to reverse transcription. The primer anneals to the polyadenylated 3`end of the RNA, so that the cDNA is synthesized and with the use of reverse transcriptase prologs into the RNA 5`end direction.

The RNA is a stencil for the RT, in order to create the determined complentary DNA, through the polymerase activity (Freeman et al. 1999!). The completed cDNA can be subjected into the polymerase chain reaction (PCR).

Real Time Quantitative-Polymerase Chain Reaction (qPCR)

The PCR contains three processes. Initially denaturation occurs by heating the sample till the very high temperature of (94-98°C), in order to destroy the hydrogen bonds and base stacking interactions, which are responsible for holding the DNA strands together. Afterwards the temperature sinks to 48-71°C, in an attempt to allow annealing the primer to the complementary regions of the template. During the extension step (68-72°C) the primer is extended by the polymerase, with the purpose of forming a getting DNA. The final part of the process lasts for 5-10 minutes in a constant temperature of 68-72°C, till finalization of all partial copies and release from every replication machine. Conclusively the temperature is lowered to 4-10°C till the tubes can be taken out (Freeman et al. 1999).

III. Results

Fluorescence-assisted cell sorting (FACS)

1.Comperison of PBMCs between ICU patients and cardiac stabile patients

Annexin V/Propidium Iodide(PI) staining

PBMCs had been prepared for the FACS analysis to compare apoptosis between PBMCs of ICU and stabile patients. We marked the cells with calcium depended phospholipid binding protein Annexin V. Annexin binds to Phosphatidylserine (PS), which is normally located in the intracellular side of healthy cells, but in death cells PS migrates to the extracellular side, where Annexin is able to bind with(eBioscience). PI is a dye that binds to DNA, and thus can be used to mark death cells (eBiolegend). Apoptotic cells are possessed by DNA fragments where PI bind on (Riccardi et al. 2006). The average of Annexin and PI stained cells was analyzed and the numbers of the both groups were dated. Experiments revealed an increase in apoptosis of cells, which were from ill patients of the ICU, contrary to healthy PBMCs (Figure: 8). Dot pots can be seen on the figure (9,10).

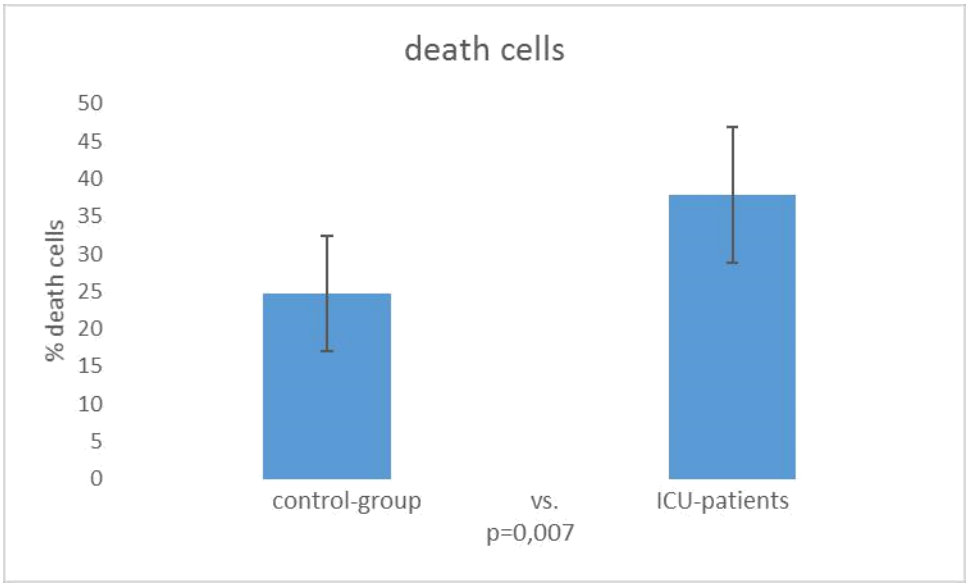

Figure 8. Comparison of death cell average between ICU and stabile patients.

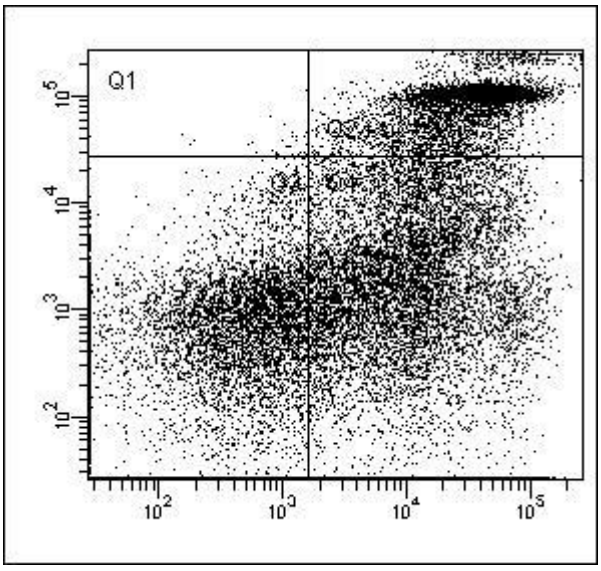

Figure 9.Dot-plot of PBMCs from the healthy control group.

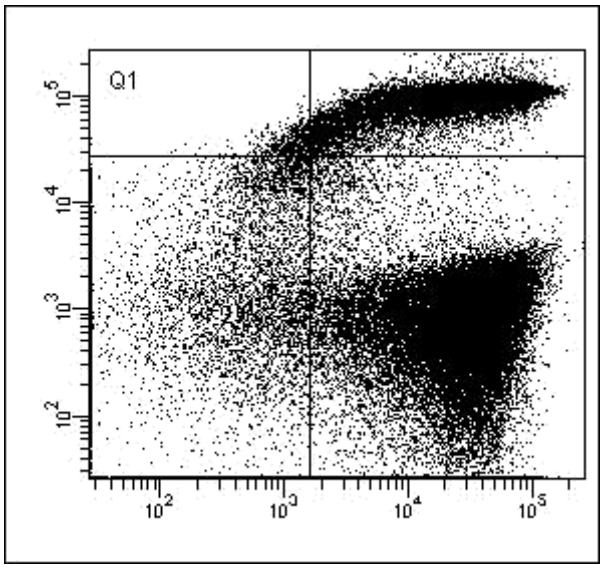

Figure 10:Dot-plot of PBMCs from ICU patients.

p53 is one of the main players of the apoptotic pathway in mammalian cells. Thus we tried to analyze the occurrence of p53 in mononuclear cells. One part of the cells possesses a higher number of p53 activation level and the other part fewer. The total number of p53 high cells was significantly increased in ICU patients (Figure 11). Also the both histograms (Figure 12,13) show us the increase of p53high cells.

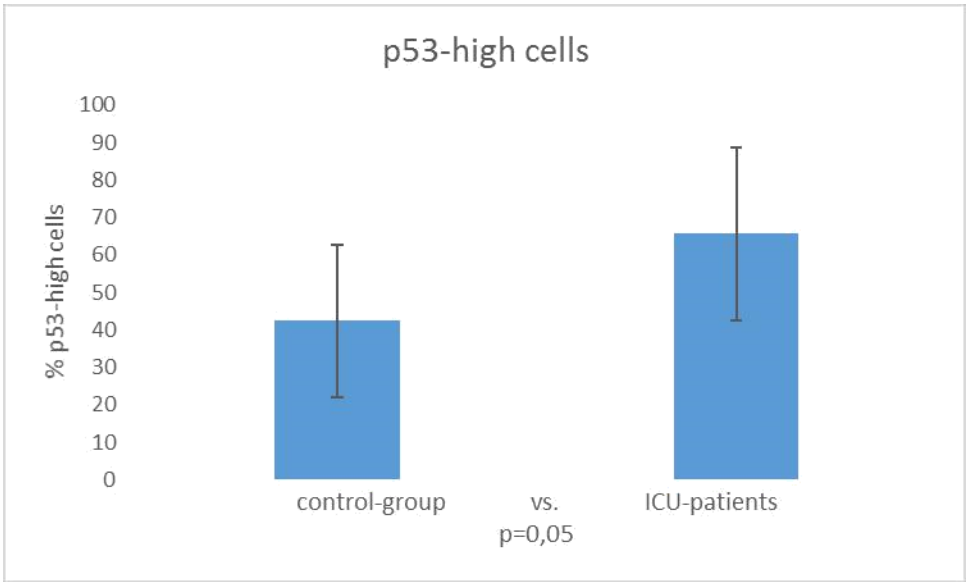

Figure 11: Comparison of p53high average f ICU-patients and the control-group.

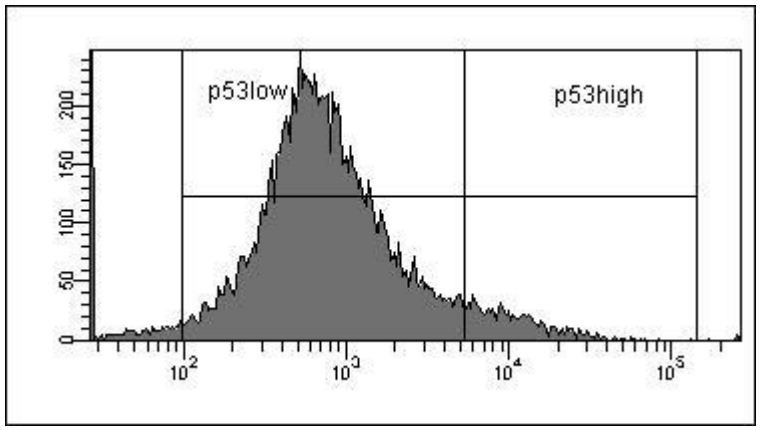

Figure 12: The figure shows clearly the p53low level of the control group.

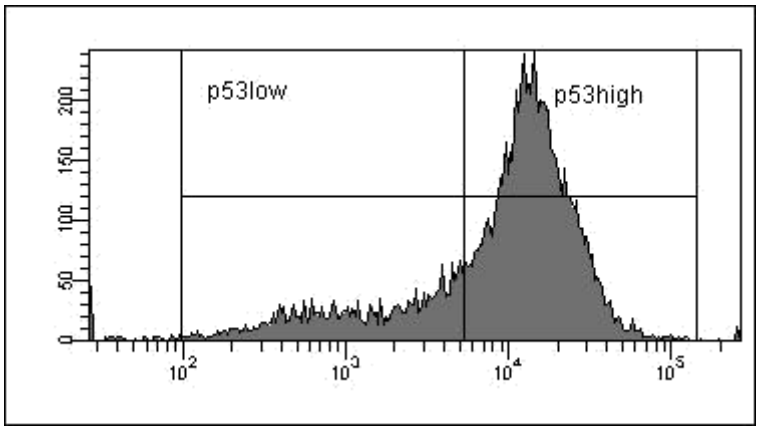

Figure 13: This figure shows the p53high level in ICU PBMCs.

p53 activity and gammaH2AX activation goes hand in hand. GammaH2AX is marker of DNA double strand breaks (Bonner, William M. et al. 2008). H2AX is a member of the histone protein family H2A, which gets phosphorylated, after DNA damage is induced. For example, through ionization radiation or cytotoxic agents. A conjugated antibody against H2AX was used to make a flow cytometry possible. Experiments show that H2AX activation increases with the number of p53-high pathway in contradiction to p53-low acting way where the number of the histone protein decreases (Figure 14). Dotplots of the experiment are below (Figure 15,16).

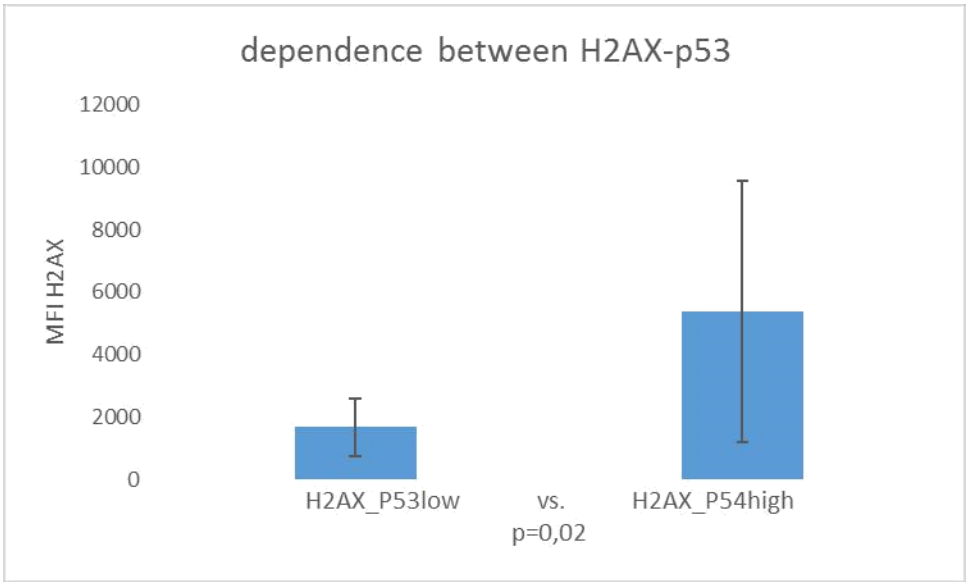

Figure 14. Comparison between the activation H2AX concerning p53 high and low.

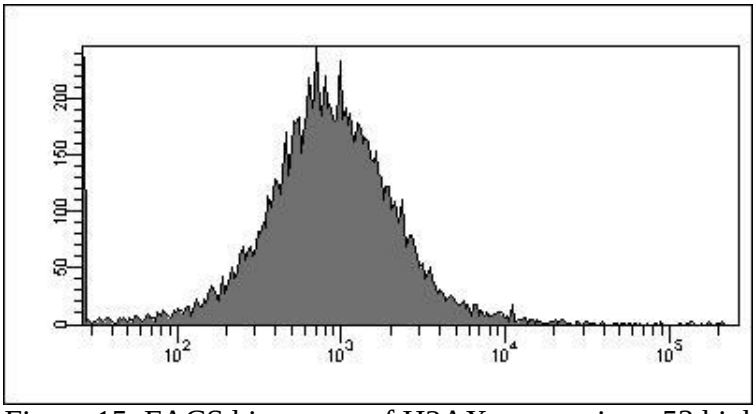

Figure 15. FACS histogram of H2AX concerning p53 high activity.

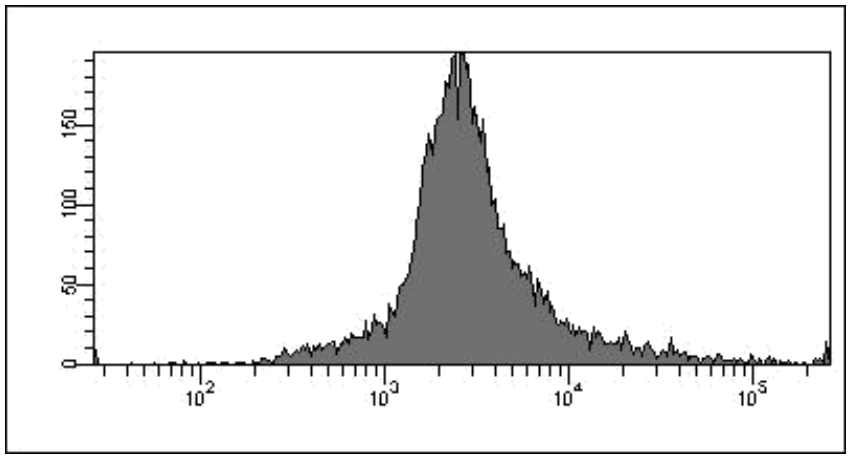

Figure 16. FACS histogram of H2AX concerning p53-low activity.

2. Comparison of apoptosis and p53 activation between ICU serum pre-treated PBMCs and healthy human serum pre-treated PBMCs

Apoptosis

It is shown that PBMCs, which were treated with ICU serum, had a higher percentage of apoptosis (Figure 17). FACS dot pots show clearly the results (Figure 18, 19).

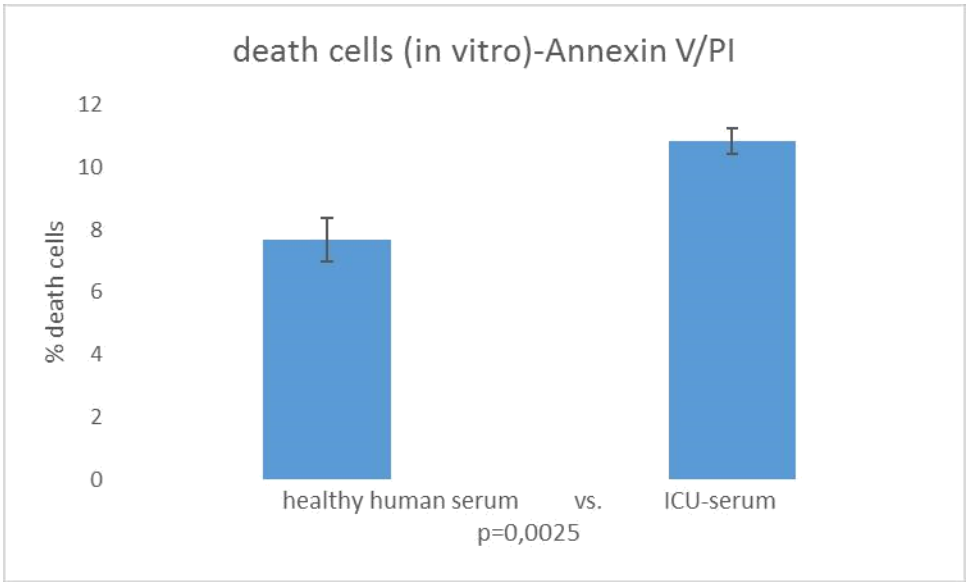

Figure 17. Comparison of PBMCs (ICU serum vs. healthy human serum) after staining with Annexin and PI.

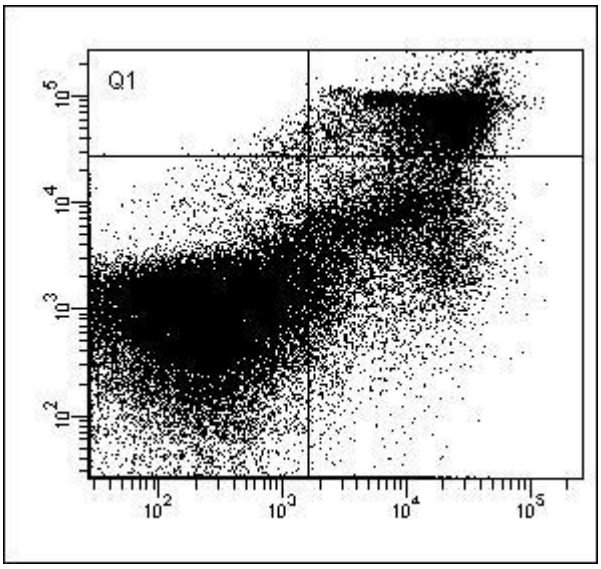

Figure 18. Dot-plot of healthy human serum pretreated PBMCs with Annexin/PI.

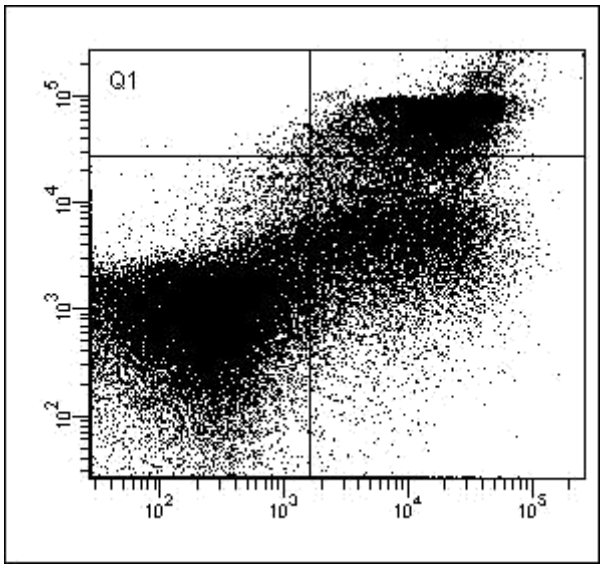

Figure 19. Dot-plot of ICU serum pretreated PBMCs with Annexin/PI.

Annexin staining without PI

It is shown that PBMCs, which were treated with ICU serum, had a higher percentage of apoptosis (Figure 20). FACS dot pots show clearly the results (Figure 21, 22).

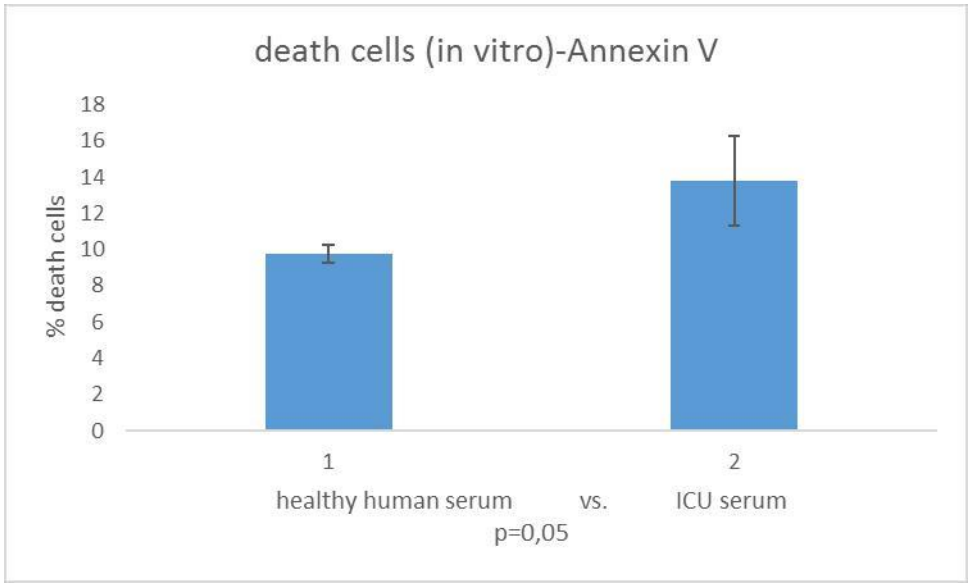

Figure 20: Comparison between ICU serum vs. healthy human serum pretreated PBMCs.

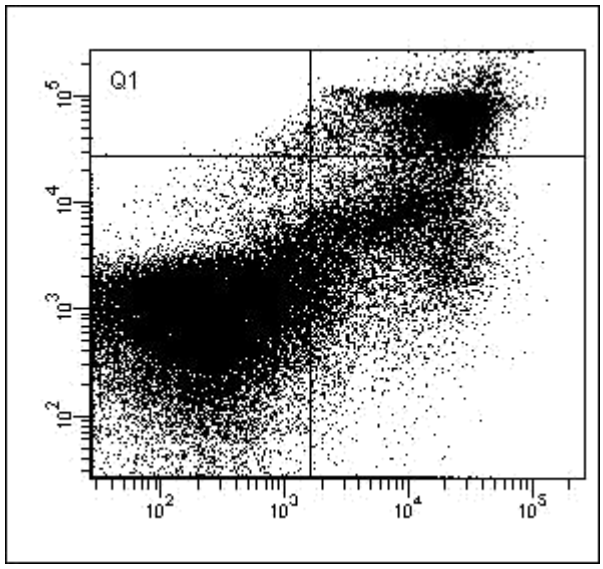

Figure 21. Dot-plot of healthy human serum pretreated PBMCs with Annexin/PI.

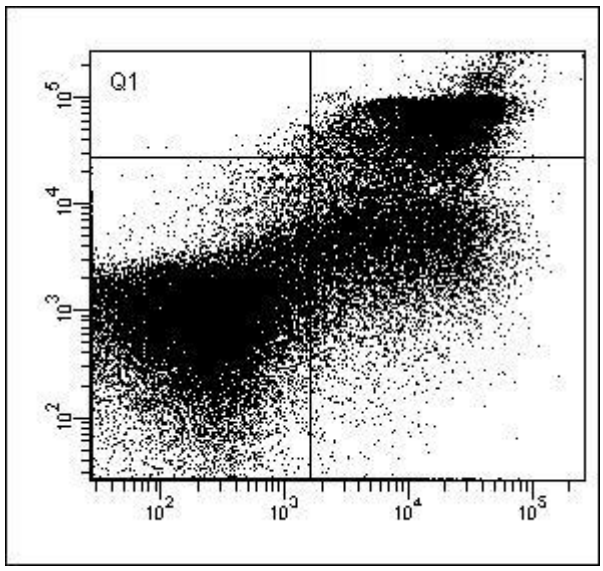

Figure 22: Dot-plot of ICU serum pretreated PBMCs with Annexin/PI.

Propidium iodide staining:

Experiments presented a higher number of PI stained cells in ICU serum pre-treated cells, than in the healthy human serum pre-treated one (Figure 23).

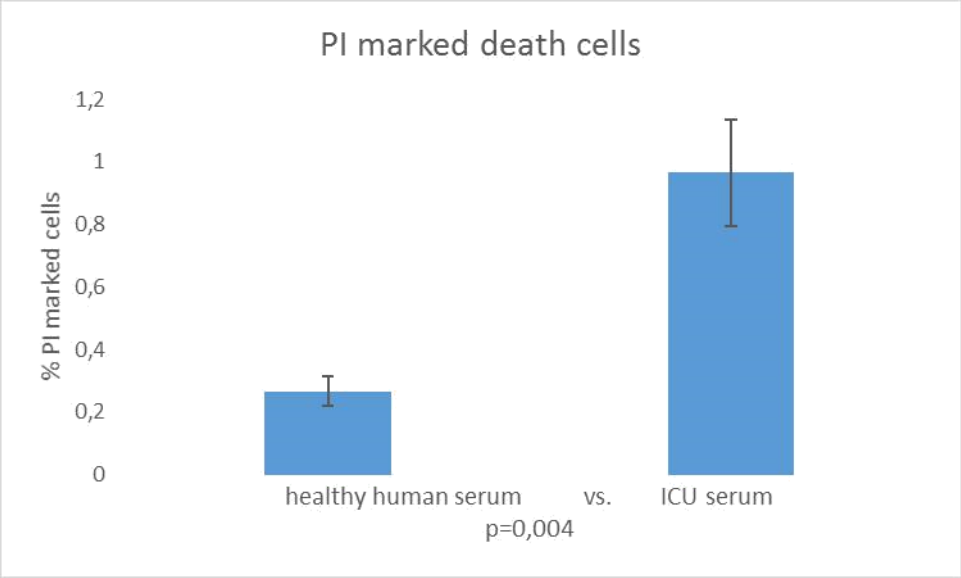

Figure 23. Comparison of annexin stained cells in healthy serum pretreated PBMCs vs. ICU pre-treated p53-inhibited PBMCs.

p53 activation

p53 is one of the main players of the apoptotic pathway in mammalian cells. The PBMCs were stained with anti CD45 antibodies to come to the percentage of p53 activation. PBMCs with pre-treated ICU serum possess a higher p53 value than healthy human serum pre-treated cells (Figure 24). Under these circumstances, we can assume that in an ill organism, the p53 is more activated. We can also show the p53 high activity of ICU pre-treated PBMCs in relation to healthy human serum pre-treated PBMCs (Figure 25,26). The experiments clearly point out the increase of p53-high activity in ICU pre-treated PBMCs.

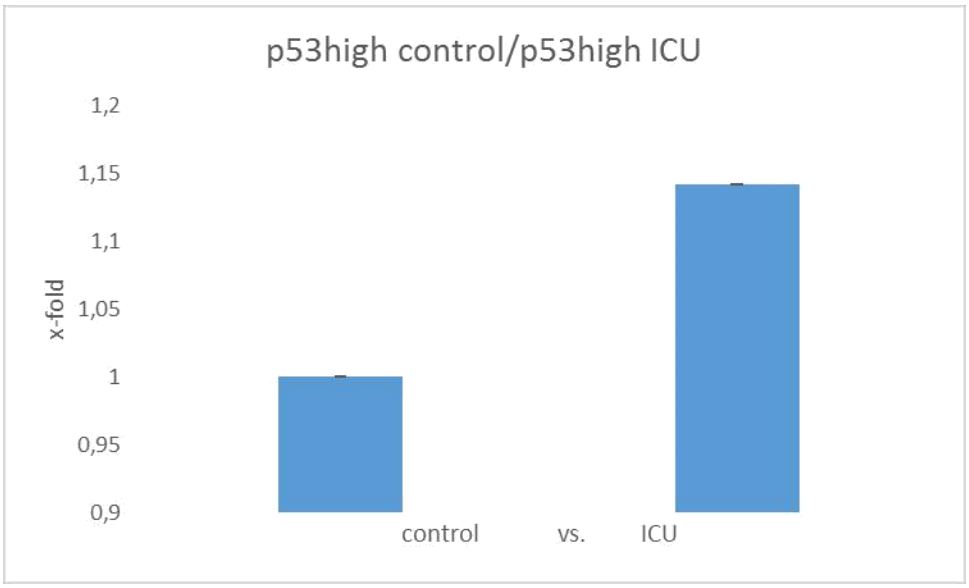

Figure 24. Comparison of p53 high activation between human healthy serum pre-treated and ICU serum pre-treated PBMCs.

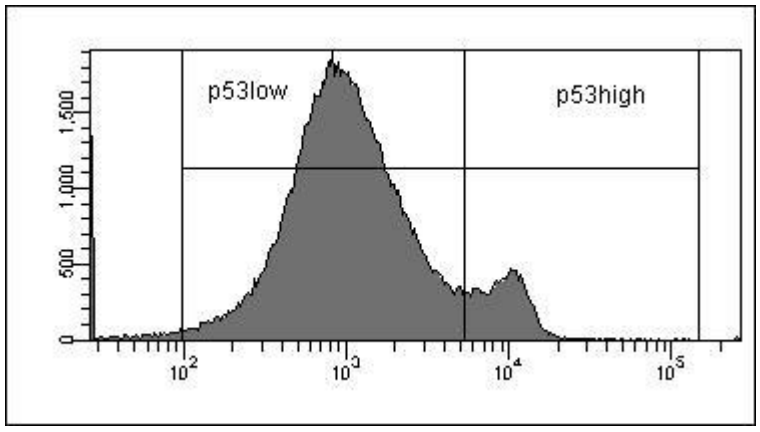

Figure 25. Histogram of p53 high and low activation in ICU serum pre-treated mononuclear cells.

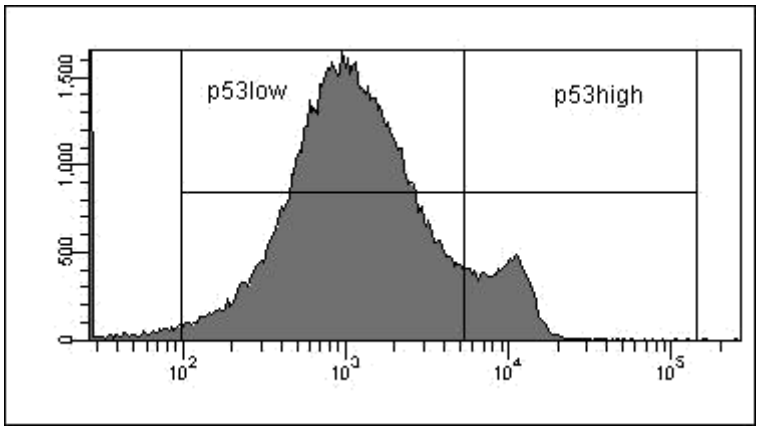

Figure 26. Histogram of p53 high and low activation in healthy human serum pre-treated mononuclear cells.

3. p53 downstream targets activation in PBMCs with ICU serum and in PBMCs with healthy serum

BAX, p53, GADD, FAS, OGG activation in p53high level cells

We could clearly see, that in both cases (ICU vs healthy human serum use) the downstream pathway of p53 is equally good activated. (Figure 27,28,29,30,31). The downstream targets BAX (Figure 27) and p53 activation (Figure 28) demonstrate a trend to more activation in ICU pre-treated mononuclear cells in comparison to healthy serum pre-treatment in contrary to FAS (Figure 30), GADD (Figure 29) and OGG (Figure 31) which were higher activated after healthy human serum pretreatment.

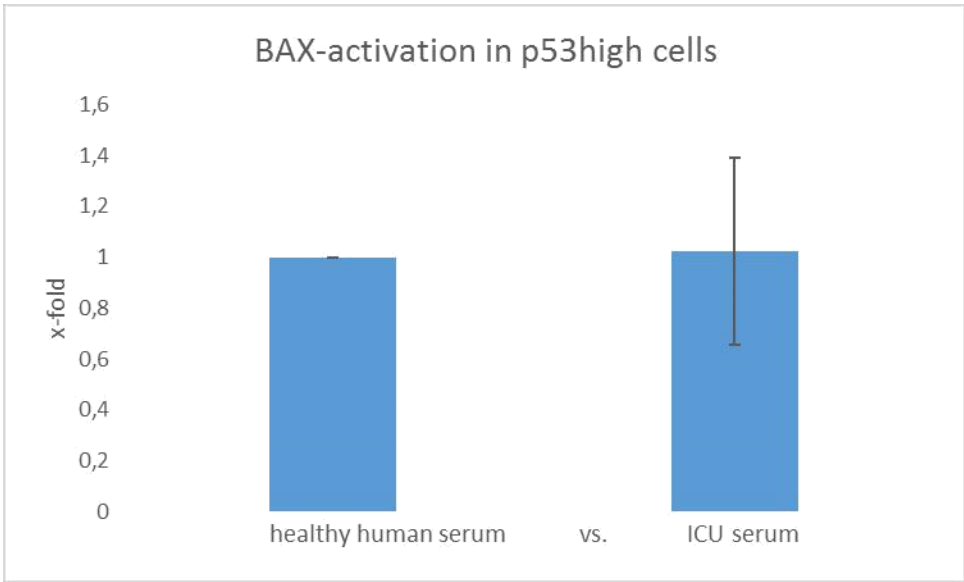

Figure 27: BAX activation in p53high cells of ICU serum pre-treated PBMCs and healthy human serum pre-treated cells.

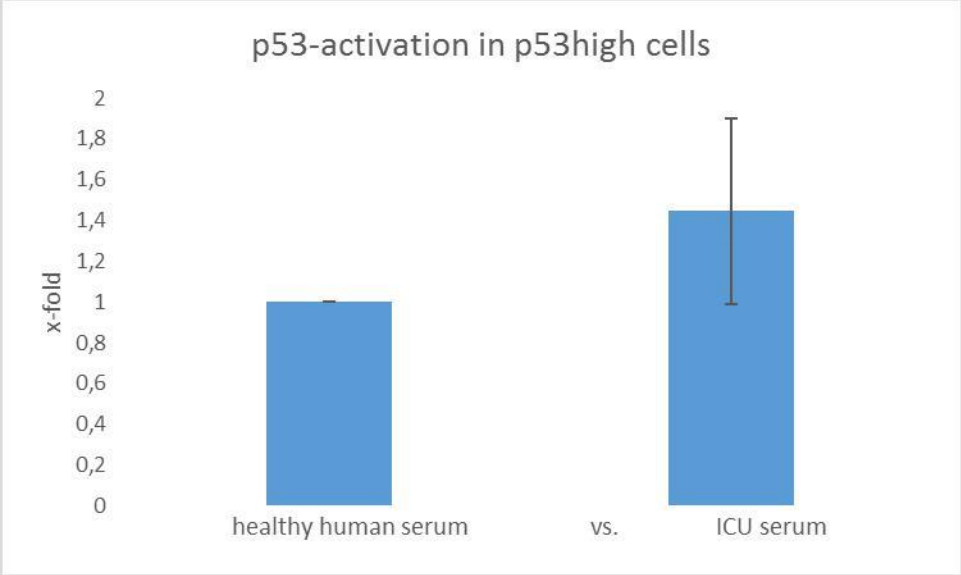

Figure 28: p53 activation in p53high cells of ICU serum pre-treated PBMCs and healthy human serum pre-treated cells.

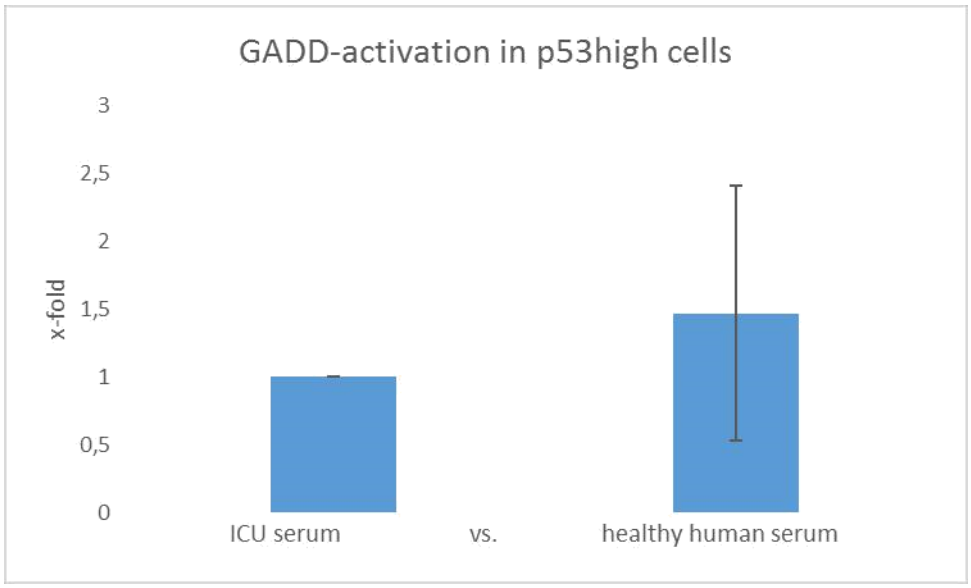

Figure 29: GADD activation in p53high cells of ICU serum pre-treated PBMCs and healthy human serum pre-treated cells.

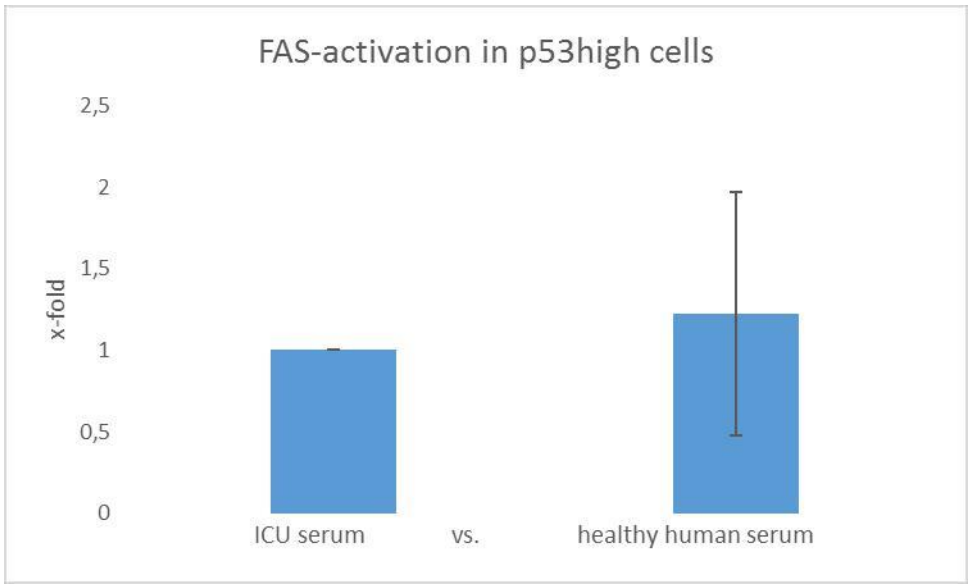

Figure 30: FAS activation in p53high cells of ICU serum pre-treated PBMCs and healthy human serum pre-treated cells.

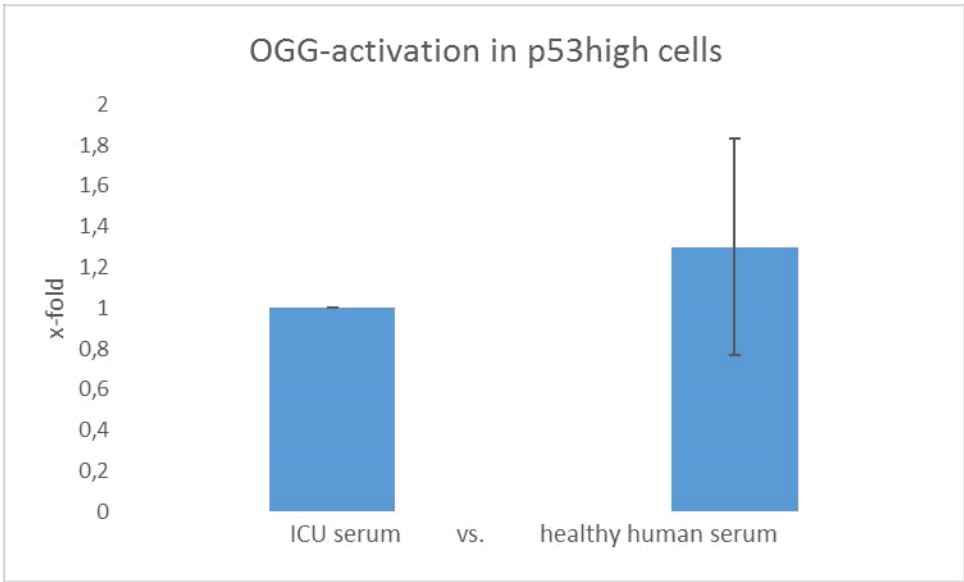

Figure 31: OGG activation in p53high cells of ICU serum pre-treated PBMCs and healthy human serum pre-treated cells.

p53-Inhibition with Pifithrin-α

For the specific experiment, we used Pifithrin, a chemical p53 inhibitor. Pifithrin blocks the transcriptional activity of p53. DMSO was used as solvent. Through p53 inhibition, we could make an assessment of the important role concerning "guardian of the genome" (Sohn D. et al 2009). Experiments show that the use of the inhibitor leads to a decrease of apoptosis in PBMCs, that did not occur in the control group. Control setups showed more apoptosis because of a functional p53 pathway (Figure 32,33,34). .

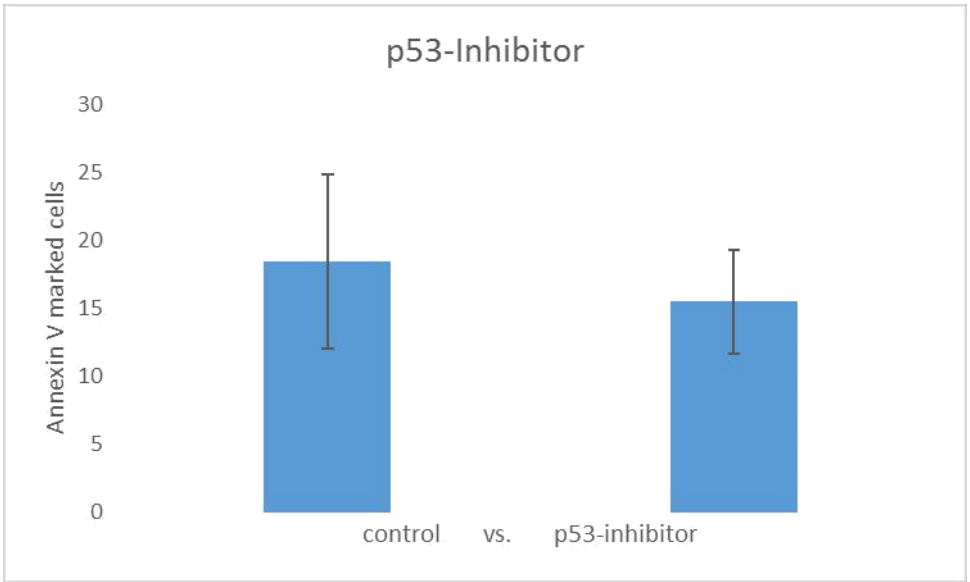

Figure 32: Comparison of apoptosis in PBMCs with inhibitor and without inhibitor.

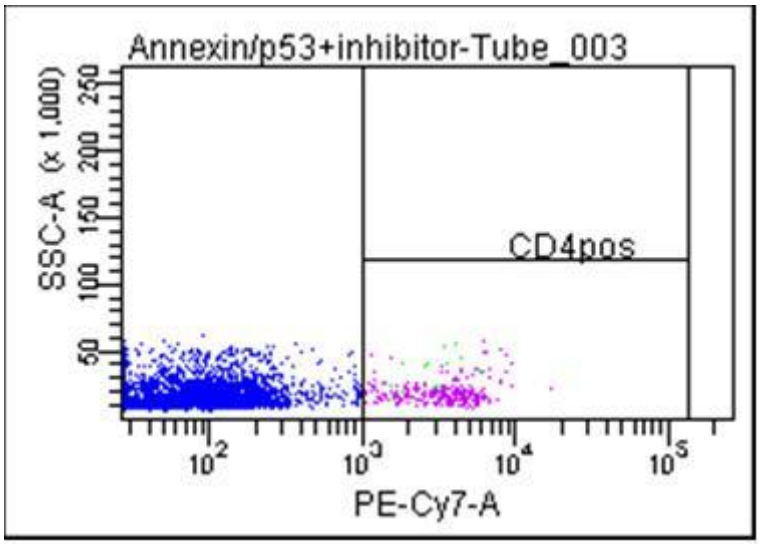

Figure 33: Dotplot with Pifithrin-α.

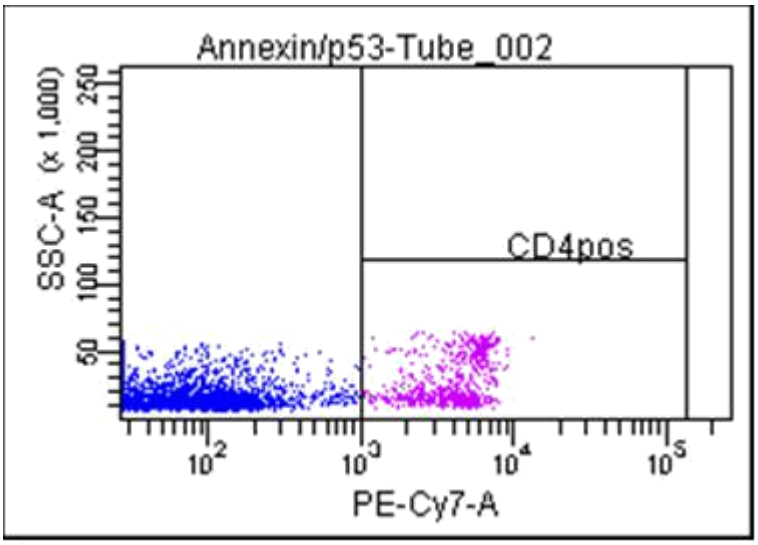

Figure 34: Dotplot without Pifithrin-α.

4. In vivo PCR-results

OGG1 activation in survivors and non survivors

When analyzing RNA expression levels of p53 between survivors (n=17) and non-survivors (n=8) in ICU patients we found no difference in p53 mRNA levels. However, increased levels of OGG1 were beneficial, as we observed a statistically significant increase of OGG1 mRNA levels in survivors compared to non survivors (Figure 33).

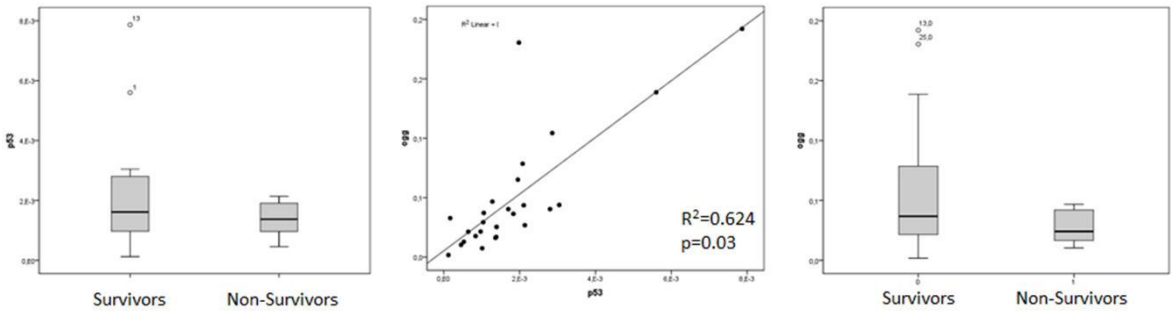

Figure 35: Increase of OGG1 mRNA levels in survivors compared to non survivors.

IV. DISCUSION AND CONCLUSION

In this thesis, both the apoptosis of immune cells in ICU patients and the main factors that play an important role in the apoptotic pathway, were analyzed.

Our findings during the practical research was: increased apoptosis average in ICU patients in comparison to healthy patients. One of the main actuators of apoptosis is p53, we demonstrated that ICU patients had a higher p53 value and the p53high activation lead to more PBMC apoptosis in contrary to p53low activity. In addition, H2AX, a marker for DNA damage, was also increased with the increased number of p53 activation. We used the p53 blocker Pifithrin-α to get out the whole contribution, and results represented the decrease of apoptosis in p53 inhibited PBMSCs. Furthermore, PBMCs with ICU serum stimulation exhibited elated apoptosis and higher p53 activation in comparison to PBMCs with healthy human serum pretreatment. Another acknowledgment was that p53 activates its downstream pathway (OGG, FAS, GADD, BAX) in the same manner and amount, both in ill and healthy organisms. Consequently, p53 plays an important role in the apoptotic induction of mononuclear cells in patients from the ICU.

Lin et al. (2007) demonstrated the same outcome with a study of thirty-nine consecutive post-operative patients from ICU and thirty healthy blood donors. The study showed clearly the increased apoptosis percentage and leukopenia in ICU patients. Patients of ICU are often confronted with SIRS, which induces lymphocyte apoptosis (Vincent 2008). Sepsis goes hand in hand with lymphocyte apoptosis (Lang 2009). The first pattern of the immunological response of sepsis, is the overproduction and distribution of pro inflammatory cytokines, like IL-1 and TNF-α, the so called “hyperinflamation”. The following process is the “hypoinflamation”, which is caused because of too little response of immune cells (Lang,

2009). To conclude with, even though proper medication and hospital treatment is applied to patients, the mortality of sepsis is certainly unaffected and cannot be decreased (Angus et al. 2001) and the apoptotic contribution to patient´s condition, the organism gets more under stress.

To sum up, both of the pathways of p53, the protective one and the apoptotic one, are important mechanisms of the human body. The downstream factors are regulated equally in ill and healthy organism. Results reveal that p53 induces apoptosis in lymphocytes and p53 high regulation is increased as well as DNA damage.

IN THE THESIS USED ABBREVATIONS

PBMC	peripheral blood mononuclear cells
AIDS	acquired immunodeficiency syndrome
TNFα	tumor necrosis factor α
FADD	Fas-associated death domain protein
DISC	death inducing signaling complex
TRADD	TNF receptor type 1-associated death domain protein
Bcl2	B-cell-lymphoma 2
APAF-1	apoptotic protease activating factor 1
DNA	deoxyribonucleic acid
MDM2	mouse double minute 2
CD 95	cluster of differentiation
DRAM	damage regulated autophagy modulator
NER	nucleotide excisions repair
GGR	global genomic repair
OGG1	8-oxoguanine-DNA glycosylase
BER	base extinction repair
ICU	intensive care unites
IL-1	interleukin-1
SIRS	systemic inflammatory response syndrome
PI	propidium iodide
BSA	bovine serum albumin
FITC	fluoresceinisothiocyanate
FASC	Fluorescence assisted cell sorting
CD4	cluster of differentiation
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
cDNA	complementary deoxyribonucleic acid

RT	reverse transcriptase
H2AX	H2A histone family, member X

USED DEVICES

BD Facscanto II (BD Biosciences) Eppendorf

Research plus pipette (Eppendorf) Microfuge

18 centrifuge (Beckman Coulter) Nanodrop

8000 (Thermo Scientific)

Allegra X-15R Refrigerated Benchtop Centrifuge (Beckman

Coulter) Eppendorf Mastercycler Pro S

LIST OF FIGURES

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ABSTRACT

Immune cells from patients from the intensive care unit are under permanent stress leading to an increased cell apoptosis. The aim of our study was to determine if p53, a key protein in cell fate, was involved in the apoptotic process.

First of all, we compared average PBMC apoptosis between ICU patients and healthy patients. Annexin V and PI were the typical markers for death cells. Cell sorting and counting was achieved with FACS. A look at the results, showed that ICU patients had a higher percentage of apoptosis than the healthy ones (control=25%, ICU=37%, p=0,007). The p53 high activity in ill patients was also increased contrary to healthy patients (control=42%, ICU=66%, p=0,05). H2AX, a histone protein for DNA damage marking, was upregulated in cells with high p53 levels indicating involvement of DNA damage (H2AX-MFI p53low=1672, H2AX-MFI p53high=5385 p=0,02).

The next setup was a different approach in the study of apoptosis of PBMCs, where one mononuclear cell colony was stimulated with ICU serum and the other one with healthy human serum. Tests demonstrated that the ICU serum pretreated PBMCs had an increased number of apoptosis next to the healthy human serum (healthy human serum pretreated=8%, ICU pretreated=11%, p=0,002). Both experiments demonstrated that the downstream pathway regulation of BAX, FAS, OGG1 and GADD was equally good for both groups. Our following goal was to inhibit p53. The use of p53 inhibitor Piphitrin α, revealed a decrease of apoptosis in p53 blocked cells (apoptosis without blocker=18%, apoptosis with blocker=15%).

Our results underpin the hypothesis of increased apoptosis value in ICU patients, project that the p53 activity is increased and more stimulated in these patients and define that the ICU serum leads mononuclear cells to more apoptosis and p53 activity.

KURZFASSUNG

Die Abwehrzellen von Intensivstation-Patienten sind erhöhtem Stress ausgesetzt, welches zu einer erhöhten Anzahl an Apoptose führt. Das Ziel unserer Studie war es, den Einfluss und den Grad der Mitwirkung von p53 im apostolischen Prozess zu bestimmen.

Zuerst, wurden die durchschnittliche Apoptose in mononuklearen Zellen in ICU Patienten und Gesunden bestimmt und verglichen. Annexin V und PI waren die typischen Marker für tote Zellen. Die Differenzierung der Zellen wurde mit Hilfe von FACS geleistet. Unsere Ergebnisse zeigten eindeutig, dass Patienten auf Intensivstationen einen höheren Prozentsatz an Apoptose haben im Vergleich zur Kontrolle (Kontrolle = 25%, ICU = 37%, p = 0,007). Die p53high Aktivität bei kranken Patienten im Gegensatz zu gesunden Patienten erhöht (Kontrolle = 42%, ICU = 66%, p = 0,05). H2AX, ein Histon-Protein für die Markierung von DNA-Schäden, stieg mit der der erhöhten p53high Aktivität (H2AX-MFI p53low = 1672 H2AX-MFI p53high = 5385 p = 0,02).

Im nächsten Setup, wurden ein Teil der Mononuklearen Zellen mit gesundem Serum und der andere Teil mit ICU Serum stimuliert. Tests zeigten, dass die ICU Serum vorbehandelten PBMCs im Gegensatz zu den mit gesunden Humanserum vorbehandelten PBMCs, eine erhöhte Anzahl von Apoptose zeigten (gesunden menschlichen Serum vorbehandelt = 8%, ICU vorbehandelte = 11%, p = 0,002). Experimente zeigten, dass Co-Faktoren von p53, BAX, FAS, OGG1 und GADD für beide Gruppen gleich gut war aktiviert wurden.

Die Verwendung des p53-Inhibitors Piphitrin α, zeigte eine Abnahme der Apoptose in p53 blockiert Zellen (Apoptose ohne Blocker = 18%, mit Apoptose Blocker = 15%).

Unsere Ergebnisse untermauern die Hypothese der erhöhten Apoptose in Intensivpatienten, mitunter die erhöhte p53-Aktivität und dass das ICU Serum die p53 Aktivität und die Apoptose stimuliert.

Curriculum Vitae

Personal data

Name	Nimet ERGÜN

Education

since 10/2010	Diploma Studies in Pharmacy
	University of Vienna, Austria
June 2010	Matriculation examination

Working areas

since 01/2014 Apotheke zur Mutter Gottes, 1100 Vienna

04/2013-11/2013 Aesculap Apotheke, 1030 Vienna

Personal skills

Computing	MS Word, MS, Excel, MS Power Point
Languages	Turkish (native speaker), German (fluent), English (good)

Wien, 06/2016

Nimet Ergin