# Diplomarbeit

# Effect of methylene blue and nicotine on the secretion of antimicrobial peptides and neurotransmitter by peripheral blood mononuclear cells

zur Erlangung des akademischen Grades

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# Inhalt

# 1 Danksagung

Ich möchte mich bei all jenen bedanken, die mich bei der Realisierung meiner Diplomarbeit fachlich oder persönlich unterstützt und gefördert haben.

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# 2 Zusammenfassung

Mononukleäre Zellen des peripheren Blutes (PBMCs) sind ein Teil der Hauptakteure der Immunologie und haben einen enormen Einfluss auf den gesamten menschlichen Körper. Neben bekannten Funktionen, wie Antikörperregulation und Phagozytose, besitzen sie viele andere Fähigkeiten, wie die Freisetzung von Antimikrobiellen Peptiden sowie verschiedener Neurotransmitter.

Methylenblau ist das erste vollständig synthetisierte Medikament der Geschichte. Sein medizinischer Werdegang begann 1891, heutzutage wird es neben anderen Indikationen zur Unterstützung bei der Therapie von Harnwegsinfekten verwendet.

Der süchtig machende Effekt von Nikotin auf den menschlichen Körper ist der Hauptgrund für die Popularität des Rauchens. Neben den intensiv erforschten Eigenschaften auf das Gehirn gibt es viele, bis jetzt noch unerforschte Effekte auf das periphere System.

Bislang wurde der Einfluss dieser Substanzen auf mononukleäre Zellen des peripheren Blutes im speziellen in Hinblick auf die Sekretion Antimikrobieller Peptide und Neurotransmitter kaum untersucht.

Die vorliegende Arbeit analysiert den potentiellen Effekt von Methylenblau und Nikotin auf die PBMC Sekretion von Antimikrobiellen Peptiden und Neurotransmitter. PBMCs wurden mittels Zugabe von Methylenblau oder Nikotin für 24 Stunden stimuliert. Anschließend wurde die Sekretion der Antimikrobiellen Peptide LL37, S100A8/A9 und Alpha Defensin-1 sowie der Neurotransmitter Adrenalin, Noradrenalin, Dopamin und Serotonin mit Hilfe von sandwich- oder competitive-ELISA Technik bestimmt.

Methylenblau wies einen auffallend stimulierenden Einfluss auf die Sekretion des Antimikrobiellen Peptides S100A8/A9 auf, sowie einen feststellbaren Effekt auf die Serotoninsekretion. Die Ausschüttung von Dopamin durch PBMCs wurde durch den Einfluss von Nikotin erhöht, andererseits wurde die Freisetzung von Noradrenalin durch Nikotin gesenkt.

Die Ergebnisse leisten einen Beitrag zum Verständnis der Wirkungsweise von Methylenblau, des ältesten komplettsynthetischen Medikamentes. Die Resultate enthüllen ebenso die Konsequenzen des Nikotinkonsums auf die peripheren Blutzellen.

# 3 Abstract

Peripheral mononuclear blood cells (PBMCs) are one of the key players in immunology and have a tremendous influence on the whole body. Beside the well-established function of antibody management or phagocytosis, there are many other functions of these cells such as secretion of antimicrobial peptides and even several neurotransmitters. PBMCs can be influenced by drugs or compounds present in the blood. In this study, the influence of methylene blue and nicotine on these immune cells was observed.

Methylene blue was the first completely synthesized drug in history. It started its medical career in 1891 and to date it is used amongst other indications as backup medication for urinary tract infections.

The main reason for the popularity of smoking is the hooking effect of nicotine on the human body. It induces beside its intensive investigated force on the brain many different so far uncharted effects in the peripheral system.

To date, the effect of these substances on peripheral blood mononuclear cells is barly investigated, especially with respect to secretion of antimicrobial peptides and neurotransmitters.

The current work analyses potential effects of methylene blue and nicotine on PBMC secretion of antimicrobial peptides and neurotransmitters. PBMCs were stimulated by methylene blue or nicotine for 24 hours. Afterwards, the secretion of the antimicrobial peptides LL37, S100A8/A9 and alpha defensin-1 and of the neurotransmitter adrenaline, noradrenaline, dopamine and serotonin was assessed by sandwich or competitive ELISA technique.

Methylene blue leads to increased S100A8/A9 secretion by PBMCs, as well as a noticeable effect on the serotonin secretion. The release of dopamine was remarkably increased by nicotine, while the same substance revealed inhibiting effects on the noradrenaline release.

Findings reported here contribute to our understanding of a further mode of function from methylene blue. Results also present the consequence of nicotine consumption on the peripheral blood cells.

# 4 Introduction

Paul Guttmann and Paul Ehrlich from Berlin,Germany, have been in the first in medical history to treat malaria patients, a very problematic infectious disease in that century, with a completely synthesized drug. The substance they chose was methylene blue, an industrial thiazine dye to heal malaria patients. (1) This was the start of methylene blues medical career. It was used for many different diseases as the review “Lest we forget you—methylene blue..” from Schirmer et al explains. In this review methylene blue is also indicated for urinary tract infections, whereas the IARC Monographs of the WHO leads methylene blue as an antiseptic therapeutic substance. (2, 3) Today in clinical daily application methylene blue is used for the treatment of inherited methemoglobinemia, acute methemoglobinemia, Alzheimer´s disease, Ifosfamid-induced neurotoxicity, vasoplegic adrenaline-resistant shock and in special cases for pediatric malaria. (2) The most important effects of methylene blue in this indications are the inhibition of monoamino oxidase, of guanylat cyclase and nitric oxide synthase as well as the reduction of methemoglobin. (4-6) In a study performed on rodents, methylene blue revealed a suppressing effect on NFκB activation and Th17 response as well as a lowering of the mRNA levels of inflammation factors like IL-6 and IL-1β.(7)

A further well established drug, mainly consumed as natural stimulant, nicotine came into the focus for this research. As one of the most addictive substances, nicotine in tobacco is widespread. This stress reducing and concentration improving tertiary amine has beside its central force influence on many other areas of the human body. It stimulates, similar to catecholamines the sympathetic nervous system and boosts this effect by releasing of catecholamines in the suprarenal glands.(8) This includes an impact on many organs like the heart, the kidneys, stomach and systems like the respiratory system and the gastrointestinal tract.

Peripheral blood mononuclear cells are a heterogenous cell population mainly consisting of B-cells, T-cells, dendritic cells as well as natural killer cells and mononuclear phagocytes generated from monocytes. As part of the unspecific and the adaptive immune response they achieve an important function in the defence

against pathogenic impacts. (9) For over ten years, the impressive effect of the secretome from peripheral blood mononuclear cells has been emphasised in many studies. It has revealed promising regenerative effects on acute myocardial infarction (AMI), stroke, cutaneous wound healing and spinal cord injury (10-15) In 2016, Kasiri et al.published a study that investigates the antimicrobial activity of the secretome from irradiated and non-irradiated human peripheral blood mononuclear cells.(16) The study shows, the secretome of both fractions includes antimicrobial peptides like cathelicidin, calprotectin, S100A9 and others. This demonstrated another way of natural immune defence of blood cells against antimicrobial burden. These substances show harmful potential against different bacteria types but are also partly involved in cytotoxic functions, angiogenic pathways or promotion of inflammation factors and others. (17-19) The research group of Univ. Prof. Dr. Hendrik Jan Ankersmit MBA analyzed the fascinating characteristics of the secretome from peripheral blood mononuclear cells. This promising secretome starts now with a phase two study for its authorization as a medicinal product for the healing of cutaneous wounds in diabetic patients.

Previously in 1994, Bergquist et al. analysed lymphocytes in relation to catecholmine uptake, synthesis and its paracrine and autocrine function.(20) This finding has been evaluated by a highly sensitive capillary electrophoresis assay. They proved the potential of lymphocyte, especially CD4+ T-cells to produce catecholamines. Further studies confirmed these results. They assumed that these released catecholamines, a type of neurotransmitters, act on common target spots as well as dose dependent paracrine or autocrine inhibitors of lymphocyte proliferation and cytokine production. (21) Catecholamines have many different effects in the human body, especially in relation to the well-known “fight or flight” response. With their sympathomimetic function, catecholamines like dopamine, noradrenaline or adrenaline, influence the heart rate and the contractility, the blood vessels, the bronchial smooth muscles, the gastrointestinal tract as well as the radial muscles of the eye and other areas. (22)

Serotonin also known as 5-HT, is another neurotransmitter, basically produced in the brain and in the gastrointestinal tract. It is important for the pain perception, for the sleep-wake-cycle, mood, and appetite and also for different functions in the respiratory, gastrointestinal and cardiovascular system. (9, 23, 24) Studies detected, that activated B-cells and mature dendritic cells are able to secret serotonin after

uptake by serotonin transporter. This secretion leads to a proliferative effect on the T-cells. (25, 26) O´Connell et al. observed in 2006 the potential of T-cells for synthesis of serotonin with detection of the 5-HT producing enzyme tryptophan hydroxylase type 1 in naive T-cells. (27) This fact has been proven by immune-labelling and fluorescence microscopy by León-Ponte et al. in 2007. (28) This demonstrates the participation of 5-HT in the adaptive immune response.

Thus, the question about a connection between the constitutional effect of methylene blue, the medical influence of nicotine and the components of the secretome from peripheral blood mononuclear cells arose.

Methylene blue has time- and dose- dependent effects on circulating blood cells and influences transendothelial migration, the release of NO synthases and of NO binding partners which are important factors for vasodilatation. (5) PBMCs are influenced by nicotine in focus on their cytokine expression and their cytolytic activity. (29) In particular the effect of nicotine on TNF secretion by macrophages differentiated from PBMCs, is an important outcome. Nicotine inhibits by a cholinergic receptor the release of TNF and in a turn the inflammatory response.(30)

In a human mixed lymphocyte reaction nicotine inhibits the expression of adhesion molecules, cytokine production and T-cell proliferation.(31) CD4 cells reduce the Th17 expression after nicotine treatment. This reveals another anti-inflammatory effect caused by the same $\alpha$7 nicotine receptor.(32)

To date, no former studies analysed the effect of methylene blue and nicotine on the peripheral blood mononuclear cells especially with respect to their secretion profile. The influence of these substances in focus on the secretion of antimicrobial peptides and neurotransmitters was not discussed or examined until now.

This investigation was designed to analyse the influence of methylene blue in different concentrations and the effect of nicotine also in different concentrations on the secretion of the antimicrobial peptides LL37(cathelicidin), S100A8/A9(calprotectin) and defensin alpha-1 as well as the secretion of the neurotransmitters adrenaline, noradrenaline, dopamine and serotonin from peripheral blood mononuclear cells. The assumption is that both substances cause a change in the release of antimicrobial peptides and neurotransmitters compared to cells without

treatment with a special substance. This would explain beside the already revealed functions additional impact of methylene blue and nicotine in case of consume.

The following subchapters highlight the background of the utilized substances and the treated cell type in view of history, function and meaning for the human body. The chapter material/methods describes the utilized science tools and the study process and gives information about the statistical analysis. In the results section, the graphical evaluation and the description of the revealed data is presented. To interpret the results the chapter discussion is attached.

# 5 Background

## 5.1 Methylene blue

### 5.1.1 History

In 1876, Heinrich Caro, a chemist from Germany, synthesized methylene blue for the first time. (33) One year later, his company patented the use of methylene blue as a tar-dye. In **1882** Robert Koch used this notable “dye” for his discovery of the tubercle bacilli. (34) Several years later, in **1891**, Paul Guttmann and Paul Ehrlich described observations made treating two malaria patients successfully with this thiazine dye (1). This was the beginning of methylene blues medical career, which is still ongoing. Thus methylene blue was the first full synthetic drug in history. The specific harmful effect of methylene blue on parasites and bacteria was one thought Paul Ehrlich educed from his studies with methylene blue as staining substance. Within this framework, methylene blue was proper for the beginning of modern drug research. Incidentally, this is the reason why until World War 1 the terms “drug” and “dye” were used as synonym.(2) Especially for the exploration of neuronal structures, the in **1896** found methylene blue method of Cajal was an important concept to visualize the collateral spines of Purkinje cells. In the beginning of Cajals dendritic research he worked with the Golgi staining method. Many sceptical colleagues did not believe in his neuronal discovery. He used different staining types, some of them developed and improved by himself, like the methylene blue method, to fortify his discovery. Subsequently Cajal and his contemporaries improved different staining methods with methylene blue to explain the majority of neuronal system from the brain to the peripheral parts. This brilliant research of the nervous system made him in 1906, together with a colleague a Nobel Prize laureate. (35) Furthermore, it is an important and inherent part in histology staining methods. As one of the two main ingredients of the famous Giemsa solution, methylene blue played an historical part in pathological analysis.(36) The in **1902** found Giemsa solution is the standard staining for malaria diagnosis and for the lymphoma classifying in the Kiel classification. It visualises histoplasma, leishmania, toxoplasma and pneumocystis and brings with high solution in dying of chromatin, nuclear membranes and of different cell components a great

requirement for microbiological work. Methylene blue is as content of the Giemsa solution part from the appraisal of helicobacter pylori and blood samples. (37) In its beginning as well as today its especially used as a dye in different industrial application areas, so for example as photoreducible dye in galvanic cells, in laboratories for cell and tissue staining or as redox indicator for iodometry and titanometry, as dye for paper and office supplies or for silk colours. (38) Beside its use in a wide range of hygienic and medical indications, methylene blue also is added to psychiatric medications. (39) The listed famous researchers are only a few of many important historical scientists and Nobel laureates that made methylene blue part of their studies.

### 5.1.2 Biochemistry

As a tricyclic phenothiazine drug, methylene blue undergoes a catalytic redox cycle. It is a blue cation, which can be reduced by nicotinamide adenine dinucleotide phosphate (NADPH) or thioredoxin.(2, 40) The reduced substance is the uncharged colourless LeucoMB. Via $O_2$ methylene blue can be re-oxidised. (Figure 1) The hydrophilic Methylene blue is not efficient to stain red blood cells in contrast to similar substances like new methylene blue or azure B. Paradoxically, methylene blue´s intracellular effect against Plasmodium spp. in erythrocytes is the highest compared to its congeners. (40) Methylene blue is partly metabolized in the body into LeucoMB and N-de-methylated metabolites (especially azure B) as well. (41) Further, the treatment with methylene blue leads to a blue urine, that could affect compliance. (42)

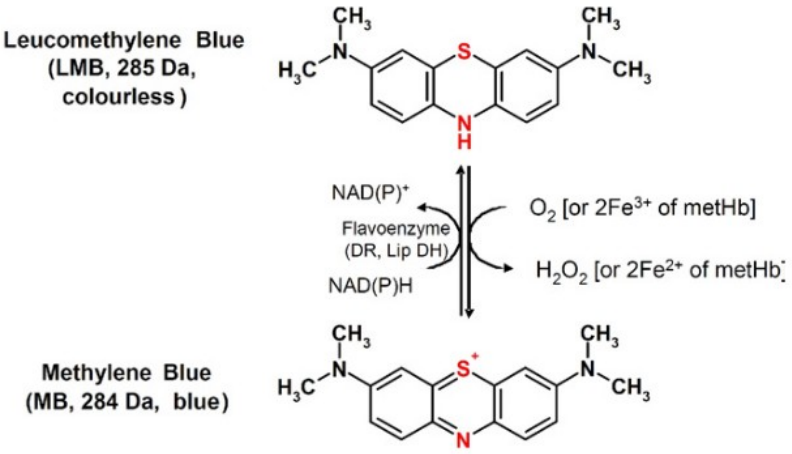


**Figure 1** **Redox-cycle of methylene blue in vivo.**
**Methylene blue is reduced by NADPH into the colourless leucoMB. In presence of $O_2$ or iron(III)- containing compounds, methylene blue will be reoxidized. In this reaction cycle NADPH and $O_2$ are consumed and hydrogen peroxide is formed (after H. Schirmer et al. (2))**

## 5.1.3 Methylene blue as a current drug

Methylene blue is a big issue in the actual medical research. Over 4000 papers have been published in the last 5 years, and this is only the result of simple PubMed research. In the review “Lest we forget you – methylene blue” its documentated that the US Food and Drug Administration (FDA) indicated in 2011 methylene blue for the treatments of enzymopenic hereditary methemoglobinemia and acute acquired methemoglobinemia as well as prevention of urinary tract infections in elderly patients. (Table 1)(2, 43)

**Table 1**
**Dosage of MB in different clinical conditions**
**(after H. Schirmer et al. (2))**

| Therapeutic indication | Dosage of methylene blue |
|---|---|
| Inherited methemoglobinemia | 1 x 50-250 mg/day (for a lifetime) |
| Acute methemoglobinemia | 1-2 x 1.3 mg/kg (i.v. over 20 minutes) |
| Ifosfamid-induced neurotoxicity | 4 x 50 mg/day p.o. or i.v. |
| Prevention of urinary tract infection in elderly patients | Orally 3 x 65 mg/day |
| Vasoplegic adrenaline-resistant shock | 200 mg i.v. over 1 hour followed by infusion (0,25-2 mg/kg/hour) |
| Alzheimer´s disease | 3 x 60 mg/day |
| Pediatric malaria | 2 x 12 mg/kg p.o. for 3 days |

Key: i.v., intravenous; MB, methylene blue; p.o., oral.

Also the paper from O´Leary et al. (44), defines the use in intraoperative visualization of nerves, nerve tissues, and endocrine glands as well as of pathologic fistulae (2). Especially the neglected methylene blue treatment of malaria patients samples a comeback in the last few years. In combination with dihydroartemisinin/piperaquine methylene blue was highly efficacious for preventing P. falciparum transmission, without difference in adverse events (excluding blue urine) to the treatment with the same medication but without methylene blue (42). Further medical indications are the prevention or treatment of ifosfamid-induced encephalopathy or neurotoxicity in cancer patients(45), at priapism (46) as treatment of choice, the treatment of intractable prurius ani (47) or against acute catecholamine-refractory vasoplegia by inhibition of guanylate cyclase (48). A big triumph was the treatment of inborn enzymopenic methemoglobinemia, colloquial named as the Blue People of Troublesome Creek (43). This hereditary deficiency in the erythrocyte diaphorase, signed by a congenital cyanosis, needs methylene blue as activator substance for methemoglobin reductase to reduce methemoglobin. (49) After treatment with methylene blue, the blue skin colour caused by the stored methemoglobin also disappeared. Little wonder that it's called "a visible success" of knowledge-based medicine (2).

### 5.1.4 Clinical application

Methylthioninium chloride "Proveblue" is the clinical pharmaceutical form from methylene blue available to use in practice. It is indicated for acute symptomatic treatment of medicinal and chemical products-induced methaemoglobinaemia. As solution for injections with a concentration of 5 mg/ml, the dosage of 1-2 mg per kg applied over 5 minutes is recommended. A revision dosage can be applied one hour after the first application. The accumulative maximum permissible dose should not exceed 7 mg/kg, because this could lead to a methemoglobinemia. The accumulative maximum permissible dose in case of aniline or dapsone induced methemoglobinemia should not exceed 4 mg/kg Proveblue. For long term infusion, the clinical data are insufficient.

Contraindications are hypersensitivity, patients with glucose-6-phosphate dehydrogenase deficiency, absence based on the risk of hemolytic anemia. Further

contraindications are treatment of nitrit induced methemoglobinemia while treating a cyanid intoxication, treatment of methemoglobinemia induced by chlorat intoxication and a lack of nicotinamide adenine dinucleotide phosphate (NADPH). The expected terminal half-life in case of injection is about 26.7 hours.(50)

## 5.2 Peripheral Blood Mononuclear Cells (PBMCs) and their secretome

### 5.2.1 PBMCs

Peripheral Blood Mononuclear Cells are that part of the peripheral blood, containing, as the name describes, the mononuclear cells. In detail these are the lymphocytes, including B-cells, T-cells, dendritic cells, NK cells, and mononuclear phagocytes generated from monocytes.

As part of the unspecific immune response, the phagocytosis practicing cells, in detail the polymorphonuclear leucocytes in the blood and the mononuclear monocytes and macrophages in blood and tissue, are the main cells of the congenital unspecific defence. After invasion of bacteria or penetration of foreign bodies in the human body, the unspecific defence eliminates them immediately by phagocytosis. This works by the release of inflammation mediators like Interleukin-1 of tumour necrosis factor-alpha. They recruit the local macrophages in the tissue and activate the endothelia to attract more leucocytes via selectins and platelet activating factor. In the step of diapedese the leucocytes penetrate the vascular wall and then move to the inflammation by chemotaxis. After the arrival of macrophages or neutrophil granulocytes at the inflammation location, they start with phagocytosis. They bind the microorganism on the cell membrane and then enclose them with pseudopodia. The harmful bacteria will be destroyed by coalescence of the macrophage including the bacteria with a lysosome. A lysosome is an intracellular component, including lysosomal enzymes like protease, peptidase, lipases and more, which kill the bacteria. Activated macrophages also synthesize defensins. These are antimicrobial peptides building ion permeable channels effecting pathogen killing. (9)

The specific cellular response is the so called adaptive immune system. It involves the antigen presenting dendritic cells, which processes antigen and presents them in

the secondary lymphatic organs to activate lymphocytes. There are different kinds of T-lymphocytes. The activated TH1-helper cells are part of the CD4+-T-helper cells. They secrete activating factors for macrophages, to support their intracellular cell killing work. The TH2-helper cells, also part of the CD4+-T-helper cells, are important for the T- and B-cell proliferation and in consequence for the immunoglobulin formation. The CD8+-cytotoxic-T-cells are stimulated by professional, antigen presenting cells. After this process, they are able to start apoptosis in viral infected cells by T-cell enzymes like perforines and proteases like granzymes. They also start apoptosis in as foreign detected cells, which causes problems after organ transplantation. The T-memory cells act via CD44 surface proteins to ensure a faster defence in case of re-infection with the same pathogen. (9)

PBMCs can be separated by different methods. The most common one is the Hypaque-Ficoll method. This separation process is based on the ficoll concentration and the different densities of the blood components. Ficoll is a sucrose polymer whose density has to be increased by Hypaque or Isopaque. Hypaque is a mixture from 3,5-diacetamido-2,4,6-triiodobenzoate with N-methylglucamine 3,5-diacetamido-2,4,6-triiodobenzoate. (51) After centrifugation of the Hypaque-Ficoll solution layered with heparinized blood with 800g for 15-20 minutes in low acceleration and low deceleration, the blood components are separated. On the bottom of the centrifugation tube, the erythrocytes and the granulocytes are accumulated. The next layer is the Ficoll solution and on top of this, the so called “Buffy coat”, including the PBMCs is generated. The plasma with the lowest density forms the upper layer. (Figure 5) For this study Ficoll-Paque Plus was used with a density of 1,077 g/ml. (51, 52)

### 5.2.2 The road to the secretome

In the beginning of the twentieth century, Paul Niehans created the modern cell therapy. His approach was to rejuvenate endogen systems by injection of xenogeneic suspensions of cells provided from different organs like endocrine glands, heart, kidney, liver, intestinal mucosa and many more, with occasionally undeniable effects. Numerous further researchers picked up this approach and developed various cell treatments including autologous or allogeneic stem cells as

well as bone marrow derived stem cells and embryonic mesenchymal stem cells. (53) Even today a great number of studies are being conducted to further investigate the potential of cell therapy. Studies in small animal showed encouraging and partly eminent results in tissue regeneration. However, data from various human trials, especially in large placebo-controlled and blinded clinical studies with large patient cohorts were inconsistent and without striking clinical effects. As mentioned in a review from Beer et al. in 2016, three meta-analyses were made with conflicting results about the effect of autologous cell therapy. (53) On the one side, Fisher et al. determined a significant reduction in the risk of mortality and rehospitalisation in conjunction with stem cell therapy for heart failure. Also the global LVEF and heart failure (HF) symptoms were significantly improved. (54, 55) On the other side, Gyöngyösi et al. declared that intracoronary cell therapy does not show a significant impact on clinical events or left ventricular modification after acute myocardial infarction. (56)

Promoted progress of cell therapy development produced the concept, that the beneficial effect of cell therapy comes from the secretome they produce. (57) Particularly the secretome from cells that are stressed or undergo apoptosis seem to be more potent, as Thum et al. assumed. (58) Ankersmit et al. picked up this hypothesis and started a study about the effect of viable peripheral blood mononuclear cells (PBMCs) and irradiated apoptotic PBMCs and their cultured supernatants. He used a model of acute myocardial infarction (AMI) with rodents, with a significant reduction of infarction size by the treatment with irradiated apoptotic PBMCs in vivo. Ankersmit revealed the positive effect of the cells and their resulting secretome released from apoptotic cells. He further discovered the immunosuppressive function of the irradiated apoptotic cells induced through the release of pro-inflammatory factors as well as its impact on fibroblasts causing a pro-angiogenic effect in the bone marrow and in the AMI area. (11) Further studies focused on the impact of the secretome resulting from irradiated apoptotic PBMCs and detected its enormous effects. The secretome from apoptotic mononuclear cells aided the development of smaller lesions after stroke and supports a better neurological improvement. (59) Hoetzenecker et al successfully proved the previous shown effect, on a porcine closed chest reperfused acute myocardial infarction model. He further revealed the vasodilatory effect by NO measurement and the inhibiting effect on platelet aggregation as well as the immunosuppressive features of

an application of secretome in high concentrations via induction of CD4+ T cell apoptosis. This effect in turn revealed an anti-inflammatory effect in a CD4+ T cell-dependent autoimmune myocarditis model performed in rodents. (10, 13) The supernatant from apoptotic PBMCs also raises the secretion of elastin, collagen type III and IV, IL-8, MMP1, MMP3 and MMP9 of fibroblasts in vitro. Especially IL-8 and MMP9 play an important part in angiogenesis and different healing processes of the ischaemic heart. (13) Mildner et al revealed in 2013 the beneficial impact of PBMCs secretome in treatment of skin wounds in rodents. This study confirmed the angiogenic effect of the secretome. (12) The secretome also contains an amount of antimicrobial peptides like calprotectin, cathelicidin, S100A9 and others. This ingredients cause modulating effect on the immune defence system. (16) An attenuating effect on secondary damage after spinal cord injury in rats was investigated too; the treatment supported a better motor function after the injury. A minor cavity formation and minor axonal damage occurs via secretome treatment. (14) Beer et al characterized the composition of secretomes from non-irradiated and from irradiated PBMCs and compared them in vitro and silico.

The secretome from irradiated PBMCs consist genes for proteins that are involved in angiogenic processes as well as wound healing and leucocyte trafficking regulation. a higher amount of phospholipids, cholesterol sulfate, cholesterol, free faty acids, cholesterol esters and triglycerides, a higher amount of microparticles and a 3-fold higher number of exosomes than in the compared non-irradiated secretome is detected.

The secretome from non-irradiated PBMCs consist genes for proteins that are involved in amino acid transport and endocrine regulation, a lower amount of lipids, of microparticles and of exosomes than in the compared irradiated secretome. (60)

Due to its abundant availability, the minimal antigenicity caused by its cell-free character and its ”off the shelf utilization” in the clinical setting, the secretome is a promising substance for the use in clinics. (12)

In 2017, a secretome-based Phase I study was completed, focusing on the tolerability and safety of autologous MNC secretome (ApoSec; Marsyas I clinical trial; EudraCT Nr.: 2013-000756-17; NCT02284360). The drug named APOSEC$^{TM}$ was produced under good manufactoring practice (GMP) for topically administration on

dermal wounds. Due to the short time of the treatment, the study formed no significant difference in wound healing between the APOSEC GMP treated group and the placebo group. The secretome was well tolerated. A phase II trial is planned to prove efficacy.

Figure 2 summarizes the ingredients and the mode of actions of APOSEC™ described above and identifies possible clinical indications.

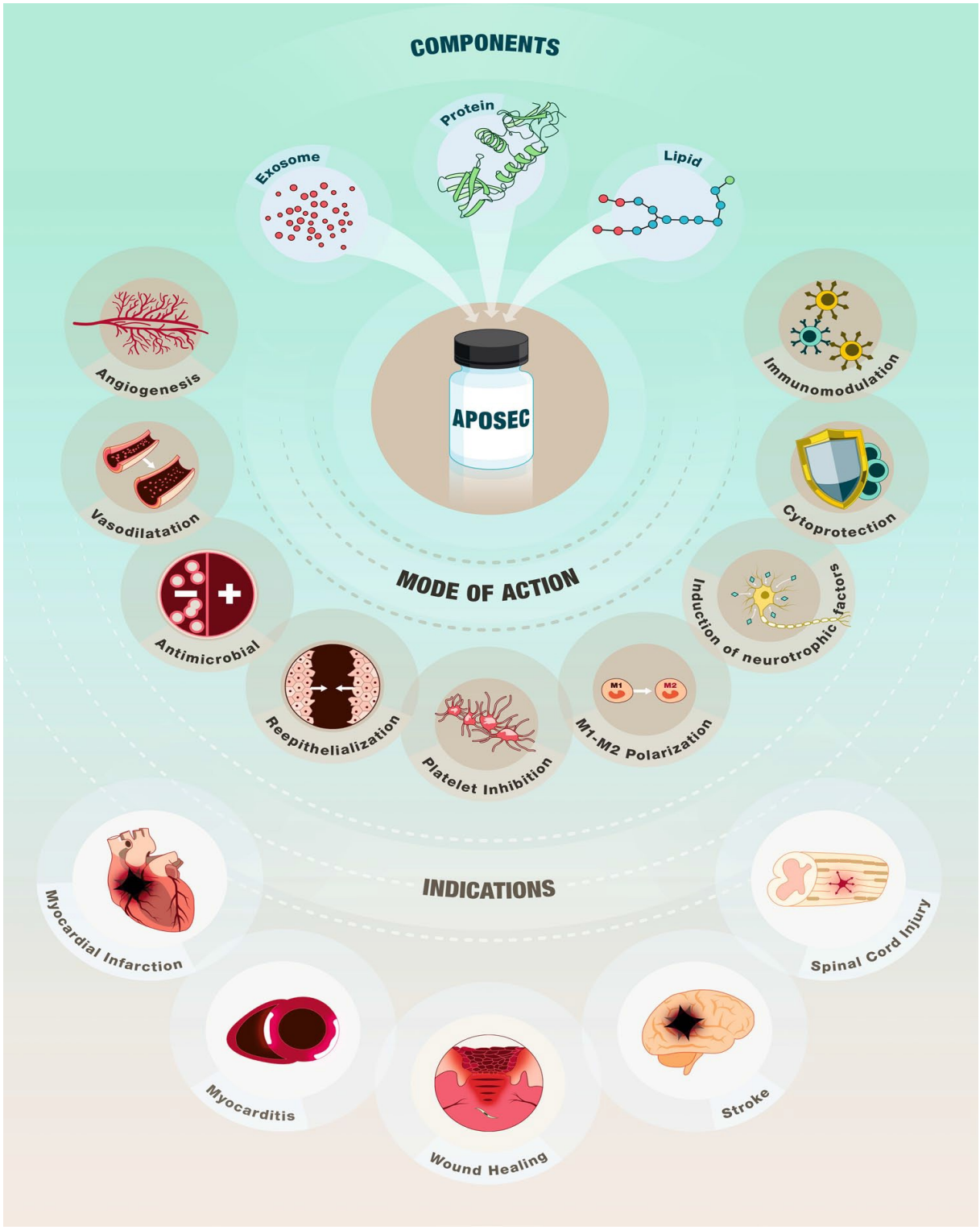


**Figure 2** **Composition of the PBMC secretome and the mode of action and its indications. The secretome is build up by paracrine substances. The best analysed ones can be distributed to specific biological groups. There are on the one side the exosomes, than the proteins and in the end the lipids. Because of the complexity of the secretome it is also likely, that other factors play an active part. The paracrine factors have shown different effects like immunomodulation, cytoprotection, induction of neurotrophic factors, M1-M2 polarization, platelet inhibition, reepithalization, vasodilatation, angiogenesis and important for this study antimicrobial function. In animal models the natural factor mixture has shown evidence on positive influence in the treatment of myocardial infarction, myocarditis, wound healing, stroke and spinal cord injury. (from Beet et al. (53))**

## 5.3 Antimicrobial Peptide

### 5.3.1 General

Antimicrobial peptides are naturally occurring substances with antifungal, antiviral, chemotactic, mitogenic and/or stimulatory functions. As part of the natural defence they are common in the mammalian immune system. The defensins and the cathelicidins are classified as the main antimicrobial peptides, acting as oxygen-independent effectors, in the neutrophils and the macrophages of the human body. (61) In 1981, the first antimicrobial peptides were described in a study from Steiner et al. The so-called cecropins have been described as effective antimicrobial substances in the blood of insects acting against pathogens. (62) Antimicrobial peptides are secreted by many different cells. For this study defensins, cathelicidin and calprotectin are the substances of interest. In 2000, Agerberth et al published a study that confirmed the expression of LL-37 (cathelicidine) and alpha defensins by lymphocytes and monocytes. (63) In 1986, the 27E10 antigen was discovered in peripheral blood and inflammatory tissue. This antigen is equal to the heterooligomer calprotectin. (64)

### 5.3.2 Defensins

Defensins are amphipathic peptides composed of 29-35 amino acids, first found in the polymorphonucleated neutrophils. (65-67) All members share 6 conserved cystein residues that take part in intramolecular disulfide bonds. These bonds are important for the antimicrobial and cytotoxic activity. (68) Defensins are variably arginine-rich. In human neutrophils the human neutrophil defensins (HNP) -1(or defensin alpha 1) ,2, 3, and 4 are found. HNP- 1, 2, and 3 are nearly identical, they just differ in their residues. (69) They are very effective killers of gram-positive and gram-negative bacteria, but more powerful against gram-positive bacteria. Low concentrations of ions like $Ca^{2+}$ and $Mg^{2+}$ enhances this effect. A high potential against fungi, especially relatives of the Candida species is documented for a wide range of defensins. (68, 70, 71) There is an effect against mycobacteria. However, M. tuberculosis has not been tested. (68, 72) Also potency from HNP-1,-2 and -3 against herpes simplex virus has been investigated in vitro, but serum and serum albumin

was able to inhibit this effect. (73) Lehrer et al. considered that the antimicrobial mechanism takes place in three stages. Stage 1: Defensin molecules or dimers pass the target cell membrane through its electromotive force. Stage 2: They perforate the membrane by building voltage-regulated pores. Stage 3: This state leads to the entry of excluded molecules and further defensins as well as loss of cell intern essential minerals and metabolites. (68) Defensins also show cytotoxic impact after 3 hours with a plateau after 8-14 hours. This effect is concentration and temperature dependant and can be inhibited by serum. (17) This activity can be increased by the presence of $H_2O_2$ in sublytic concentrations. (74)

### 5.3.3 Cathelicidins

Cathelicidins are synthesized as group of precursors. They are molecules with an N-terminal cathelin-like proregion and a C-terminal antimicrobial domain. (75) One famous representative of this group is the cathelicidin peptide LL-37, the only cathelicidin in humans. Human cathelicidin peptide LL-37 is synthesized out of its pre-protein hCAP18 by splitting-off through proteinase-3. (76) It shows antibiotic effects on gram-positive and gram-negative bacteria. The antibacterial effect of cathelicidin peptide LL37 depends on the dimension of its alpha-helical, oligomeric conformation. (77) LL37 is found in many different areas of the human body, but mainly in the mucus layers. This offers the conclusion that the main task of LL-37 is to eliminate pathogens from the human mucus layers. (78) LL-37 is part of the human unspecific immunity and beside its antibacterial effects there are many other functions. It can mediate cellular effects by binding through lipopolysaccharides and O-antigen layers on the outer membrane of bacteria. (79) Human cathelicidin peptide LL-37 also forms sizeable channels compatible with toroidal pores, that let molecules pass uncontrolled from inside or from outside, similar to the defensin function. (80) It is up to restrain the bacterial growth by halting cell wall synthesis as well as reducing inflammation. (79, 81) LL-37 also acts as factor in angiogenesis, wound healing and re-epithelialisation of the skin. (18, 82) Beside leukocytes, myelocytes and the bone marrow, LL37 is also expressed in breast milk, nails, sweat, lung epithelia, saliva, testis and others. (83) Human serum attenuates the potential harmful cytotoxic effect of LL37. (77) Further effects from LL-37 are inhibition of biofilm formation, chemotaxis, histamine and prostaglandin degranulation from mast-cells, induction of

immunmediators and suppression from apoptosis of neutrophils, but mediation of apoptosis in epithelial cells. (84-89)

### 5.3.4 S100A8/A9 (Calprotectin)

Calprotectin is a hetero-oligomer built from S100A8 and S100A9. (90) The name calprotectin originated from a study by Steinbakk et al published in Lancet in 1990. They investigated a calcium binding L1 protein with 36.5 kD, that is present in neutrophils, monocytes, certain macrophages, and mucosal and squamous epithelia as well as in serum and plasma of healthy people. It exhibited antibacterial and antifungal effects. With this in mind, they suggested the name CalProtectin, because of its calcium binding and antibacterial characteristics. (91) There are many studies with many different nomenclatures for calprotectin like S100A8/A9, MRP8/14, calgranulin, L1 or 27E10 antigen. Calprotectin adheres to cell membrane inhibiting the binding from monocytes to fibronectin or collagen. (92) It further promotes the secretion of Interleukin-6, tumour necrosis factor alpha and superoxide anions from monocytes. (19) Calprotectin is a useful inflammation marker, it is released from activated neutrophils or monocytes during the interaction of monocytes with inflammatory activated endothelium. (93) In clinical settings the calprotectin level is measured as marker for organ transplantations, pulmonary disease, rheumatoid arthritis or gut inflammation. (92) S100A8/A9 captures Zinc and as consequence influences the Zinc metabolism. (94) S100A8/A9 shows a zinc dependent antimicrobial and apoptosis inducing effect. (95, 96) In case of zinc deprivation, S100A8/A9 inhibits matrix metalloproteinases, important enzymes for wound healing, inflammation, cancer, angiogenesis, tissue destruction and embryonic development. (92)

## 5.4 Neurotransmitter

### 5.4.1 General

Neurotransmitters are chemical compounds to act as agent between nerves, synthesized in presynaptic cells or in the cells of the adrenal medulla.

Neurotransmitters are stored in vesicles next to the cell membrane. After a signal induced influx of calcium caused by an arriving action potential the neurotransmitters are secreted to the synaptic cleft. By binding to receptors at the postsynaptic cell neurotransmitters regulate ion channels. These channels change the membrane´s permeability effecting a de- or hyperpolarisation of the cell or cells. They can have an activating or a deactivating affect. The inactivation acts by enzymatic reduction or by reabsorption in the presynaptic cell or diffusion out of the synaptic cleft. Neurotransmitters that are released from the adrenal medulla are mainly reduced in the liver via catechol-O-methyltransferase or monoamine oxidase. Neurotransmitters can be classified in two groups: the biogenic amines and the amino acids. Biogenic amines are decarboxylated amino acids with diverse physiological functions (97). For this research study the biogenic amines noradrenalin, adrenaline, serotonin and dopamine are the neurotransmitters of interest.

### 5.4.2 Serotonin

Serotonin, also known as 5-Hydroxytryptamine, is mainly produced by neurons of the nuclei raphes in the middle brain as well as from enterochromaffin cells in the digestive tract, but is also found in many tissues. (9, 23, 98) For its synthesis, the essential amino acid tryptophan is needed. This acid is converted by tryptophan 5-hydroxylase into 5-hydroxy-L-tryptophan, what is the tempo defining step, and then via aromatic L-amino acid decarboxylase into serotonin. (Figure 3) It is saved in vesicles and after secretion reuptake, reduction into 5-hydroxyindoleacetic acid, or transformation into melatonin over intermediate stages is the option. (23) Serotonin has a limited ability to pass the blood brain barrier. (99) Serotonin receptors are classified in seven families that include 18 receptors spread through the whole body. Only one non-specific reuptake transporter is known. The so called SERT is a target receptor for many neuropsychiatric drugs. Serotonin is an important neurotransmitter for the sleep-wake-cycle, for pain, especially in the colon, as well as for emotional and cognitive functions. (9, 23, 24) There is also evidence, that serotonin plays an important part in neurogenesis and aging of the brain, in energy balance and in nausea and vomiting. In the gastrointestinal tract, serotonin is meant to play a role in fluid secretion, and ion transport regulation, the motility and the contraction of the internal sphincter. It is assumed, that Serotonin is included in bone formation, but the

evidence is conflicting. (23) Watts et al revealed in 2012 the connection between blood pressure and the serotonin concentration. (100) An impact of serotonin on the liver, the cardiovascular system with focus on cardiovascular events, the respiratory system, the immune system and on the platelets has been detected. (23) Serotonin is also secreted by T- and B- lymphocytes, causing, beside the direct organ effect, paracrine and autocrine effects. (25-27)

### 5.4.3 Catecholamines

There are three body´s own catecholamines called dopamine, noradrenaline and adrenaline based on the amino acid tyrosine. On the one side, neurons absorb tyrosine from the extracellular space. On the other side, they are able to build it from the essential amino acid phenylalanin. In the first building step, tyrosine is hydroxylated by the enzyme tyrosinhydroxylase to the semi-finished product 3,4-dihydroxyphenlalanin (Dopa). This enzyme appears mainly in the adrenal medulla and dissolved in the axoplasma of catecholamine neurons. It is also the speed controlling enzyme of the synthesis. After this step, the aromatic L-amino acid-decarboxylase, widespread in areas of the human body, like the liver, serotonin neurons or in the kidneys, decarboxylates dopa into dopamine which is stored in vesicles via dopamine carrier. Noradrenline and adrenaline are built out of dopamine by dopamine-ß-hydroxylase and phenylethanolamine-N-methyltransferase.(Figure 3) (101)

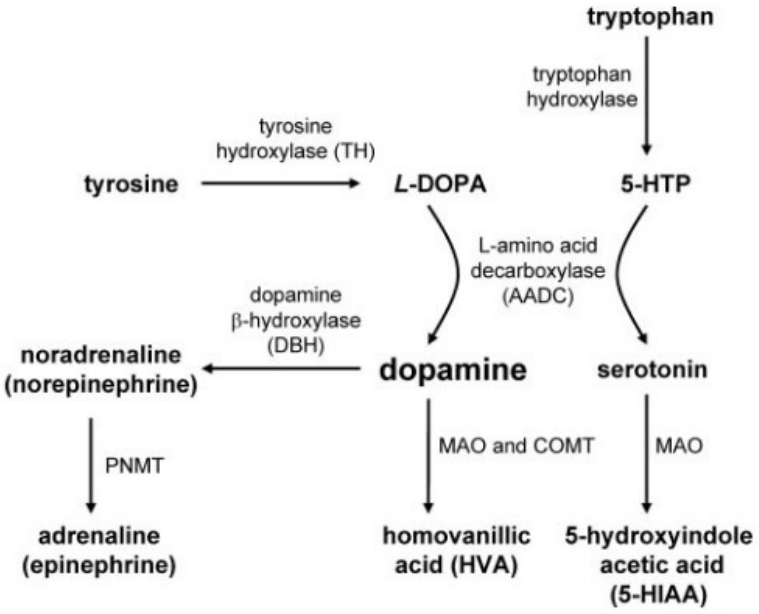


**Figure 3** **Synthesis of dopamine, noradrenaline, adrenaline and serotonin. COMT, catechol-O-methyl transferase; 5-HTP, 5-Hydroxytryptophan; MAO, monoamine oxidase; PNMT, phenylethanolamine-N-methyl transferase; (after Rubi et al. (102))**

### *5.4.3.1 History*

The canonical story from Henry Hallett Dale, an employee of the University College London describes, that in 1893/1894 George Oliver, a practicing physician from North Yorkshire, who experimented with extracts of various animal glands came to Edward Albert Sharpey Schäfer, a professor of physiology at University College London. Dr. Oliver reported him from an experiment he made on his son, by injecting a glycerine extract from calf´s suprarenal gland under the boys skin followed by a narrowing effect of the radial artery measured by an own invented instrument. This instrument was, able to measure the diameter of a living artery through the unbroken skin. Prof. Schäfer, just finishing an experiment in which he documented the arterial blood pressure of an anaesthetised dog, was sceptical. But after an injection of the extract into the veins of a new dog actually prepared for the recording of vascular effects, he finds himself standing" like some watcher of the skies, when a new planet swims into his ken´ watching the mercury rise in the manometer with amazing rapidity and to an astounding height, until he wonders whether the float will be thrust right out of the peripheral limb." (103)

This was the beginning of the understanding and research on the suprarenal gland products. Further experiments on different animals like dogs, cats, rabbits, guinea-pigs and monkeys with suprarenal extracts from the calf, from the sheep, the guinea-

pig, the cat, the dog and from humans followed. (104) Nearly at the same time, two polish authors, Wladyslaw Szymonowicz and Napoleon Cybulski, also found similar results with an important difference. They used blood from the adrenal veins for injection intravenously in a dog, what caused a hypertensive effect. In contrast, bloods from other veins didn´t show any comparable result. (105, 106) In 1905, Elliott et al. published a paper about the functions of adrenaline on different organs. In this paper he described the effect of adrenaline on the organs without any neuronal adaption, what leads him as one of the first to the statement that adrenaline works over structures derived from and on peripheral neurons. What was headed as the ´birth certificate´ of chemical neurotransmission. (106, 107) Over the century the awareness about dopamine, noradrenaline and their receptors began and grew.

#### *5.4.3.2 Receptor-mechanism*

Adrenergic receptors are expressed on the most important organs and tissues in the whole body. There are 9 different types known at the moment, categorised in three groups: the alpha-1, the alpha-2 and the beta group. These ones are further divided in subtypes. Adrenaline and noradrenaline as well as many utilized drugs unfold their potential via these receptors. All adrenergic receptors are G-protein-coupled. Adrenaline and noradrenaline have a high affinity for alpha adrenergic receptors. For beta receptors, just the adrenaline affinity is high, noradrenline´s affinity is lower, this was one of the main reasons leading to the differentiation. (22)

The alpha-1 adrenergic receptors take their main part in cardiovascular sympathetic effects due to their expression in vascular smooth muscle cells and in cardiac striated muscles. Hence, alpha-1 adrenergic receptors have a vasoconstriction and a cardiac remodelling effect. There are 3 subtypes known: alpha-1a, alpha-1b and alpha-1d which are all coupled to $G\alpha_q$ proteins. These proteins boost intracellular calcium and an activate protein kinase C. The receptors are also affine to other signalling molecules like phospholipase A2, calcium and potassium channels and small G-proteins and MAP kinases. (22)

Alpha-2 adrenergic receptors primarily are important for the pre-synaptical feedback inhibition of noradrenaline. They are also expressed in the peripheral and central nervous system, and in placenta and blood vessels, causing vasoconstriction by

circulating neurotransmitters. Alpha-2a, alpha-2b and alpha-2c as the subtypes of the alpha-2 type are different distributed through the target organs. Alpha-2a for example acts as agent for the baroreceptor reflex mechanism in the brain, leading through a lower heart rate and sympathetic outflow from the heart, caused by high blood pressure. (22) The alpha-2 types also have functions in neuro psychiatric disorders (schizophrenia, drug withdrawal, etc.), stress response, locomotion and memory. (108, 109) They are all coupled to $G\alpha_i$ proteins and they, as consequence, inhibit adenylyl cyclase. (22)

Beta adrenergic receptors especially act in the cardiovascular system and in metabolic activities like lipolysis. (110) There are three subtypes: beta-1, beta-2 and beta-3, which are coupled to Gs proteins, but they can also connect with Gi proteins. The affinity of adrenaline and noradrenaline varies from subtype to subtype. (22)

Dopamine receptors are very complex in their function. There are two types: D1-like receptor, which is coupled to Gs proteins and D2-like receptor, which is coupled to Gi proteins. These two types include 5 subtypes: D1-like family: D1R and D5R and D2-like family: D2R, D3R and D4R. D1-like receptors are also able to couple to Gq proteins and activating its cascade. D2-like receptors also have effect on ion channels with the $G\beta\chi$ subunit after separation of the G units. Dopamine receptors occur also as homodimers and heterodimers, which are able to bind further G-protein coupled receptors.

For further information about the catecholamine receptors, the review from Tank and Wong is recommended (22)

#### *5.4.3.3 Function*

Adrenaline also called as `epinephrine´ or ´suprarenin´, is mainly produced in chromaffine cells of the adrenal gland or in the “paraganglien”. As a sympathomimetic substance with special affinity to ß2-receptors, adrenaline has a huge impact on many organs and functions in the human body. Adrenaline causes a vasoconstriction on the peripheral blood vessels. The blood vessels of the skeletal muscles otherwise are going to be extended because of the predominant presence of ß2-receptors. In the human heart, ß1-receptors are dominant, so adrenaline causes a rise of the heart rate and contractility as well as a rise of the systolic blood pressure.

(111) Catecholamines are not able to cross the blood brain barrier, so the present catecholamines in the brain are synthesized there. [6] The central effects of catecholamines are elimination of tiredness, stimulation of breathing, reduction of appetite and mental agitation. Adrenaline is a substance that supplies energy immediately to the body by mobilising glycogen, gain of adipolysis and gain of $O_2$ consumption. Further effects are the reduction of gut motility, atony of the bronchial muscles and mydriasis. (112) With this in mind adrenaline is a common drug in treatment of anaphylaxis, shock, cardiac arrest and as supplement in local anaesthetic solutions. (111)

Postganglionic-sympathetic neurons, the adrenal glands as well as the central nervous system use noradrenaline as transmitter. Like adrenaline, noradrenaline stimulates the heart and causes vasoconstriction effects on the blood vessels. They also relax bronchial smooth muscles, inhibit the bronchial and gastrointestinal glands in building fluid, relax the gastrointestinal smooth muscles, the stomach muscle motility and contract the sphincters of the gastrointestinal tract. By inhibition on the secretion of insulin, increased glucagon secretion and boosted glycogenolysis, adrenaline is able to raise the glucose level in blood as well as raising the level of free fatty acids by activation of triglyceride lipase and interaction with adipocytes. Catecholamines induce mydriasis by interaction with alpha-1 receptors expressed on the radial muscles. In the kidney catecholamines increase $Na^+$ retention and stimulate the renin-angiotensin-aldosteron system. (22)

#### *5.4.3.4 Dopamine*

Dopamine is produced in sympathic nerves, adrenal medulla and non,neuronal cells, the so called amine precursor uptake and decarboxlyation cells (APUD). (22, 113) APUD cells are found in the kidney, the exocrine and endocrine pancreas, and the retina. (22) Also peripheral leukocytes are members of this group. (114) The dopamine level in the periphery increases after exercise or surgery. (115, 116) In low concentrations, dopamine causes a vasodilatation on the blood vessels. If the concentration raises an effect on the heart including increased heart rate and contractility starts, but this effect comes along with a baroreceptor reflex, leading through noradrenaline excretion from sympathetic nerves in the heart with a stimulating but also inhibiting outcome. This makes it difficult to estimate this

influence. At high, not physiological, concentrations, dopamine has a vasoconstriction effect on the blood vessels. In the proximal tubule of the kidney, dopamine increases natriuresis by binding through D-1 like receptors and by inhibiting the renal renin-angiotensin system. So the peripheral effects of dopamine are mainly vasodilatation, especially in the kidney and the mesentery, and to lower the blood pressure. Furthermore, there are peripheral effects on glucose and lipid metabolism, immune system, retina and tumour cells. (22)

## 5.5 Nicotine

Nicotine, a tertiary amine and also a weak base, is the addicting ingredient of tobacco. After smoking a cigarette, the distilled nicotine comes over carrier particles through the lungs. The nicotine is absorbed and transported over the arterial circulation through to the brain. It passes the blood brain barrier and binds in the brain tissue to nicotinic cholinergic receptors, a kind of ligand-gated ion channels. This leads to entry of cations like sodium and calcium into the cells. (8) The effect of nicotine on the brain makes the smoker feel pleasure, it is stimulating, reduces stress and anxiety. It further helps to control the mood and influences the concentration and reaction time in a positive way. (8) Next to the earlier mentioned effect of improved mood, withdrawal symptoms can be severe and lead to craving, irritability, anxiety, restlessness, concentration problems and/or impatience. (117) Nicotine has a half-life of two hours, but accumulates after smoking over six to nine hours in the human body. It is metabolized in the liver by CYP2A6, CYP2B6 and CYP2E1 enzymes to cotinine, a measurable metabolite proportional to the nicotine abuse. (118) Nicotine stimulates the sympathetic nervous system, releases catecholamines in the central nervous system and the adrenal medulla, makes vasoconstriction in the skin and the coronary arteries, and increases the heart rate and the myocardial contractility. (119-121) Nicotine impairs the insulin sensitivity, causes arterial damage and has an impact on the development of diabetes. (122, 123) After one cigarette the nicotine concentration in the peripheral arterial blood is about 26-60 ng/ml in the arteries 3-5 minutes after smoking and between 15 and 30 ng/ml in veins 5-8 minutes after smoking. (118, 124)

# 6 Material/Methode

Peripheral blood mononuclear cells have been isolated by Ficoll Paque-density gradient centrifugation from five heparinised blood donations. The samples have been acquired at the Red Cross blood donations headquarter in Linz, Upper Austria.

After isolation, the PBMCs have been incubated at a cell concentration of 25x10^6 cells/ml with methylene blue and nicotine separately in different concentrations. For methylene blue, concentrations of 25mg/L, 6,25mg/L and 1,56mg/L and for nicotine concentrations of 5µg/ml and 50ng/ml have been chosen. Cells without any special treatment served as control. In total there are 6 conditions examined within each donor. The cell free supernatant has been stored at -20°C to evaluate three antimicrobial peptides (cathelicidin, alpha defensin1 and calprotectin) and four neurotransmitters (adrenaline, noradrenalin, serotonin, dopamin) via sandwich enzyme-linked immunosorbent assay (ELISA) in further progress. (Figure 4)

## 6.1 Blood samples

For this study, five buffy coats isolated from heparinised blood, which are usually waste products in the process of blood donation, have been used. The donors for the study were randomly chosen from the Red Cross blood donations headquarter in Linz, Upper Austria. The donor information about the risk of blood donation and the further use of blood products was signed by each donor and is incumbent on the Red Cross of Upper Austria. In case of donation problems an adequate patient-centred care has been covered on site. The Red Cross provides every donor with a health-form which they are obliged to complete. [Attachment 9.2] Exclusion criteria for the study have been the common exclusion criteria for blood donation with the Red Cross in Austria like epilepsy, infections with HIV or Hepatitis-B or –C, MSM, Malaria patients, malign disease, pregnancy and others. (125) Only people aged between 18 and 60 years, with no prior history blood donations, were considered. Personal information of the donors (date of birth, sex, blood group) have been treated with care and were not forwarded to the laboratories at the Medical University of Vienna.

A unique barcode was assigned to every donor, the remaining personal data of the donors have been reserved at the Red Cross. Therefore, the staff at the Medical University of Vienna who conducted further studies was blinded and got no access to personal data of donors except the blood group and the rhesus factor.

## 6.2 Schematic representation

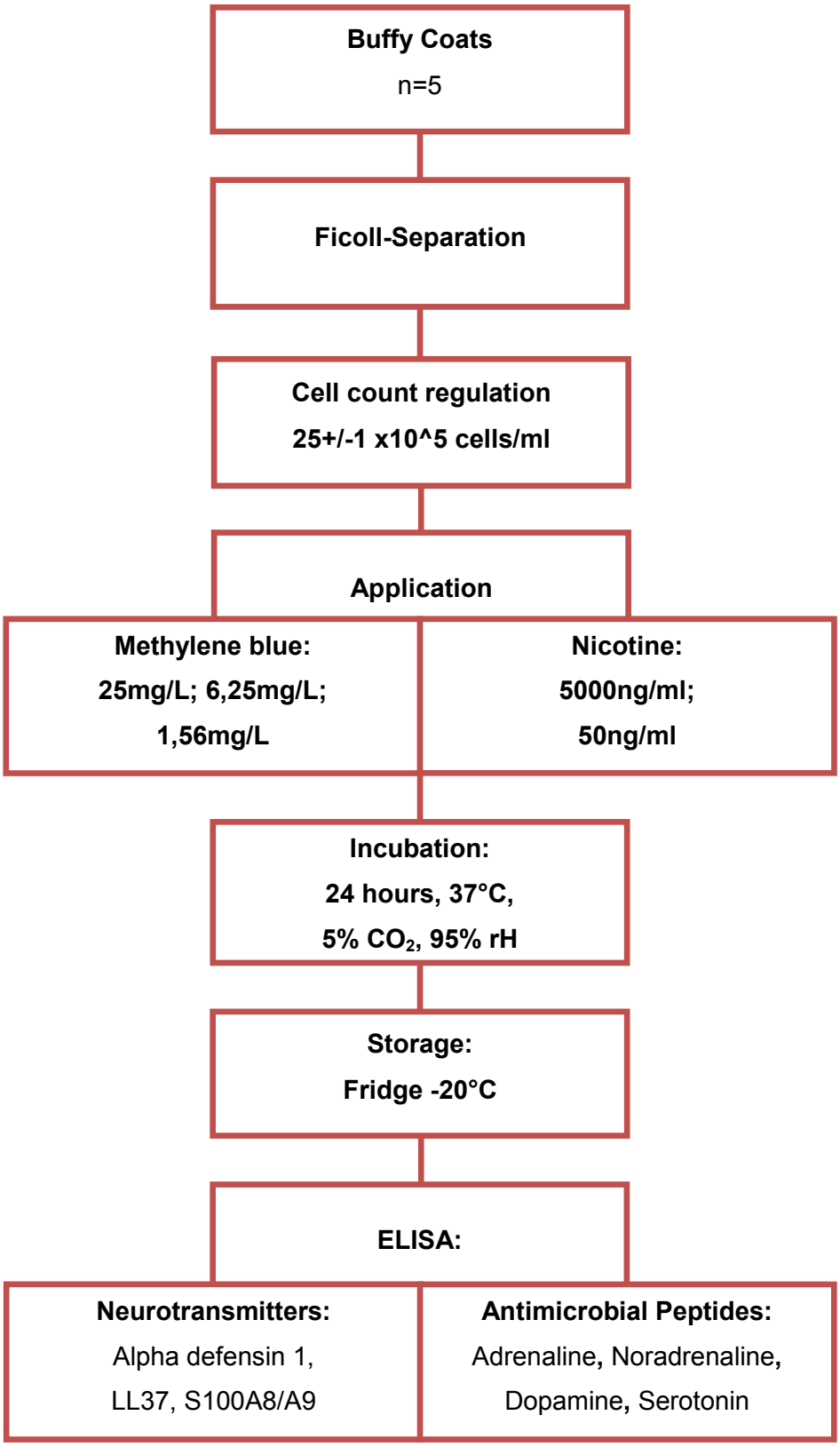


**Figure 4** **Structure of the study.**

## 6.3 Substances for treatment

For the cell treatment methylene blue in the shape of the common clinical drug solution Methylthioniumchlorid(e) Proveblue 5 mg/ml from Provepharm was used, which is intended for intravenous treatment only. This substance is indicated for the clinical treatment of methaemoglobinemia and the common dosage form for the use of methylene blue in hospitals. (50) The nicotine used in the study was a pure solution of nicotine in a concentration of 50 mg/ml (kindly provided by Assoc. Prof. Dr. Barbara Messner, Cardiac Surgery Research Laboratory, Medical University of Vienna).

## 6.4 Execution

The whole practical work has been completed in the research laboratory of the department of Surgery at the Medical University of Vienna under supervision of Univ. Prof. Dr. Hendrik Jan Ankersmit MBA and Mag. Dr. Alfred Gugerell.

## 6.5 Toxicity-assay EZ4U

The toxicity-assay EZ4U was chosen for pre-testing the possible negative impact of methylene blue and nicotine on peripheral blood mononuclear cells in different concentrations. This proliferation test is a common way to investigate the effect from cytokines, nutrients, drugs and other substances with harmful potential on different kind of living cells. EZ4U combines the benefits of the MTT-method as well as of the Thymidine-test. The MTT-method is based on the reductive potential of mitochondria in living cells. They are able to reduce the colourless up to poor coloured tetrazolium salts to strong dyed formazan derivates. The Thymidine-test works via incorporating the radioactive nucleoside, 3H-Thymidine into the cellular DNA. (126)

For this assay, serial dilutions have been prepared from methylene blue in the shape of Proveblue and from the provided nicotine solution. Methylene blue was diluted step by step in proportion 1:2 with the medium CellGenix GMP DC (CellGenix, Freiburg,Germany) resulting in dilution factors of: 1:2; :4; :8; :16; :32; :64; :128; :256;

:512; :1024; :2048; :4096; :8192; :16384; :32768; :65536;. Addition of cell suspension with a resulting concentration of 3x10^6 PBMCs/ml has been considered in calculation.

For nicotine dilution factors from 1:4x10^1 up to 1:4x10^12 increasing by a 1:10 factor have been chosen.

The self-colouring from the dye methylene blue turned out as a problem. For this reason a serial dilution of methylene blue without cell treatment has been made to subtract this disruptive effect. For 100% viability as a positive control, untreated PBMCs in a concentration of 3x10^6 cells/ml and as negative control CellGenix GMP DC medium only was used. The cells have been prepared after the following description in 5.7 and 5.8. The EZ4U assay was executed after the enclosed test instruction manual.

The Test-kit:

- Biomedica EZ4U – CELL PROLIFERATION ASSAY
    - BI-5000

## 6.6 Preparation of the treating substances

The concentration of treating substances was in case of methylene blue: 25mg/L; 6,25mg/L and 1,56mg/L, equates a dilution of 1:200; 1:800; 1:3200 in the final cell suspension. In case of nicotine, a final concentration in cell suspension of 5µg/ml and 50ng/ml (1:10^4; 1:10^6) was applied. The stock solution of methylene blue has been diluted with phosphate buffered saline (PBS) from 5mg/ml to a preparation concentration of 2,5mg/ml; 0,625mg/ml and 0,156 mg/ml which equates to a dilution of 1:2; 1:8 and 1:32. The stock solution of nicotine has also been diluted with PBS from 50mg/ml nicotine to 500µg/ml (1:10^2) and 5µg/ml (1:10^4). For further use a volume of 10µl/ml cell suspension was added to the cells. This results in another dilution of 1:100 and ends up in the planned concentration for the cell treatment.

## 6.7 Ficoll-Separation

According to the standard operating procedure of the Red Cross Upper Austria, the blood samples were processed the day after the donation. During the work flow, a blood bag with about 40 ml buffy coat (PBMC concentrate) is separated. Five of these blood samples have been sent to the laboratory at room temperature. Immediately after the receipt, the buffy coat was transferred from the bag into 50 ml tubes. Then each blood sample has been diluted proportionately 2+1 with 10 ml Hank´s balanced salt solution (HBSS). In a prepared centrifuge tube, the cell solution has been layered carefully but efficiently on 15 ml Ficoll-Paque Plus density gradient media. After centrifugation at 800g for 15min, accel: 3, decel: 1, a buffy coat consisted of PBMCs developed. (Figure 5) Buffy coats from the same donor have been carefully transferred via pipette into fresh tubes and the cell solution has been washed twice each with 50ml HBSS by centrifugation (1500RPM, 2min, accel: max; decel: max). In these washing steps, a pellet appeared on the ground. The overlaying wash solution has been discarded and the cell pellet has been resuspended in HBSS or in the end of the washing steps in 9.5ml CellGenix GMP DC medium. Taking into account the residual liquid in the tube, the final volume should be 10ml in each tube.

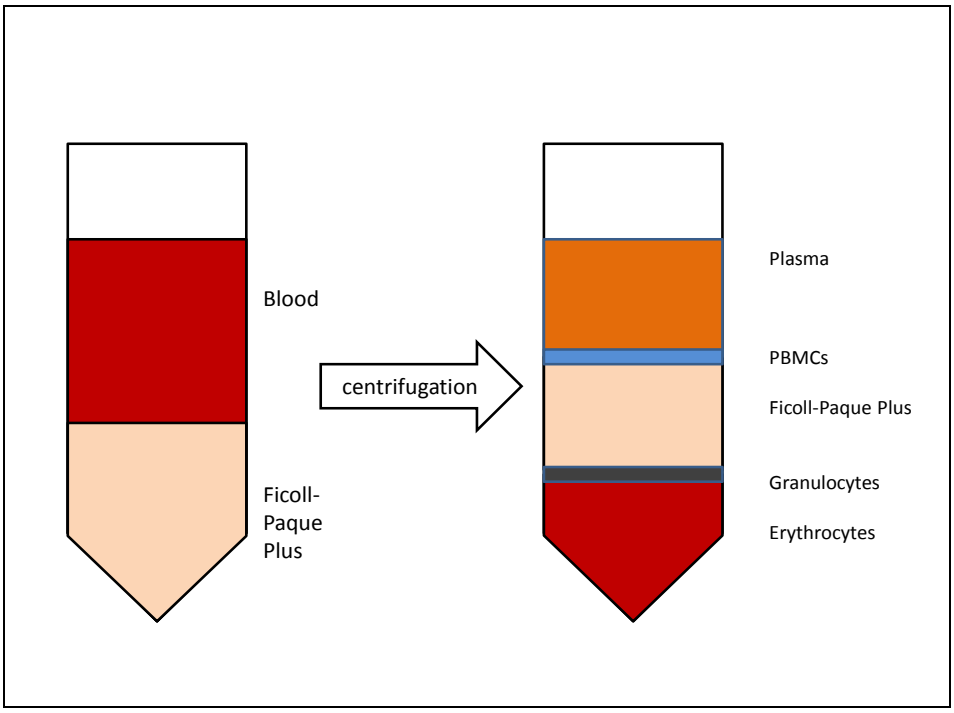


**Figure 5** **Blood separation by the Hypaque-Ficoll Method.**

## 6.8 Cell count regulation

To determine the cell count, 80 µl of the resulting cell solution have been measured with a Sysmex automated hematology analyzer. To adjust the count on the planned 25x10^6 cells/ml the following formula has been used.

*Volume (Cell count actual) x cell count (actual) = Volume (cell count target) x cell count (target)*

The needed volume has been adapted with the calculated amount of CellGenix GMP DC medium, resulting with a cell count of 25x10^6 cells/ml. After this, another Sysmex measurement has been made as control.

## 6.9 Application and incubation

In 24 well plates, 1ml adapted cell suspension per donor and per treatment concentration was placed in duplicates, each into one well. After this, the cells have been treated with 10 µl of the appropriate prepared treatment solution. The control cells have been, considering on the minimal treatment volume, left untreated. This results in 3 conditioned well plates. The treated cells have now been incubated for 24 hours in an incubator at 37°C, 5 % $CO_2$, 95% rH. After 24 hours, the content of the wells have been transferred into centrifugation tubes. The two wells with the same content has been pooled. After centrifugation (1500 RPM, 9 min, accel:max; decel: max.) the supernatant was stored in Eppendorf tubes at -20°C.

## 6.10 Ezyme-linked immunosorbent assay (ELISA)

To measure the concentration of our target substances, the neurotransmitters adrenaline, noradrenalin, serotonin, and dopamine, and the antimicrobial peptides calprotectin, cathelicidin and alpha defensin 1 in the supernatant of our treated PBMCs, we had chosen the enzyme-linked immunosorbent assay (ELISA). More precisely so called sandwich enzyme-linked immunosorbent assays.

The Test kits:

- Genway Biotech 3-CAT (Adrenaline/Noradrenalin/Dopamine) ELISA Urins
    - Catalog Number: GWB-67E01A
    - VPE 3 x 96 tests
    - LotNr: GC0032-01
    - For research use only
    - Store: 4°C Expire: 2018.12
- EIA Serotonin ELISA
    - Beckman Coulter
    - REF IM1749 RUO
    - LotNr: L102, L103, L108, L160
- DuoSet ELISA Human S100A8/S100A9 Heterodimer R&D Systems
    - DY8226-05
    - LotNr: P141740
- DuoSet ELISA Human alpha-Defensin 1 R&D Systems
    - LotNr: P139296
- Hycult Biotech Human LL-37 HK321 Edition 08-16
    - Catalog Number: HK321-01
    - LotNr: 20415K1017-Z
    - Szabo Scandic

### 6.10.1 ELISA-Background

The ELISA is a quantitative assay, based on the function of antigen and antibody binding systems. Peter Perlmann and Eva Engvall from the Stockholm University in Sweden and, at the same time Anton Schuur and Bauke van Weemen from the Netherlands found the ELISA technique in the 1970s. Since that time this assay technique has been reevaluated many times, and different variations of this immunosorbent assay have been developed, the immunozytometric tests or the different kinds of sandwich ELISAs. It also got access into clinical testing. Many important tests today are based on this antibody-antigen principle, like many virology screenings (hepatitis, HIV) or screenings to diagnose microbiological disease (toxoplasmosis for example). It is an everyday test and modern medicine cannot be

imagined without this assay. (127) In the following subchapters for this study relevant ELISAs will be highlighted.

#### 6.10.1.1 The sandwich ELISA

The normal sandwich ELISA is structured as follows. There are two kinds of antibodies. The first one, the so called capture antibody or coat antibody, has to be coated on the ground of a 96 wells plate. In some kits this is already done by the company. In the next step, the sample to investigate is placed in the well. If the sample contains the substance of interest, a protein, for example, this protein binds specifically with its antigen on the antibody. Then the sample will be removed and the wells are washed. Only the substance of interest remains bound on the coated ELISA plate. Now the second antibody, the so called detection antibody, is added to the well. This one includes an enzyme function and also binds on the substance of interest but on another antigen. After washing out of the surplus antibody, a substrate is added which changes its colour by the detection antibody equal to the amount that is left in the well binding on the substance of interest. A stop solution stops this process mostly by changing the pH and after that the extinction of light can be measured through the wells. (Figure 6) By relating the results to a standard curve, the concentration of the substance of interest can be determined. (128, 129)

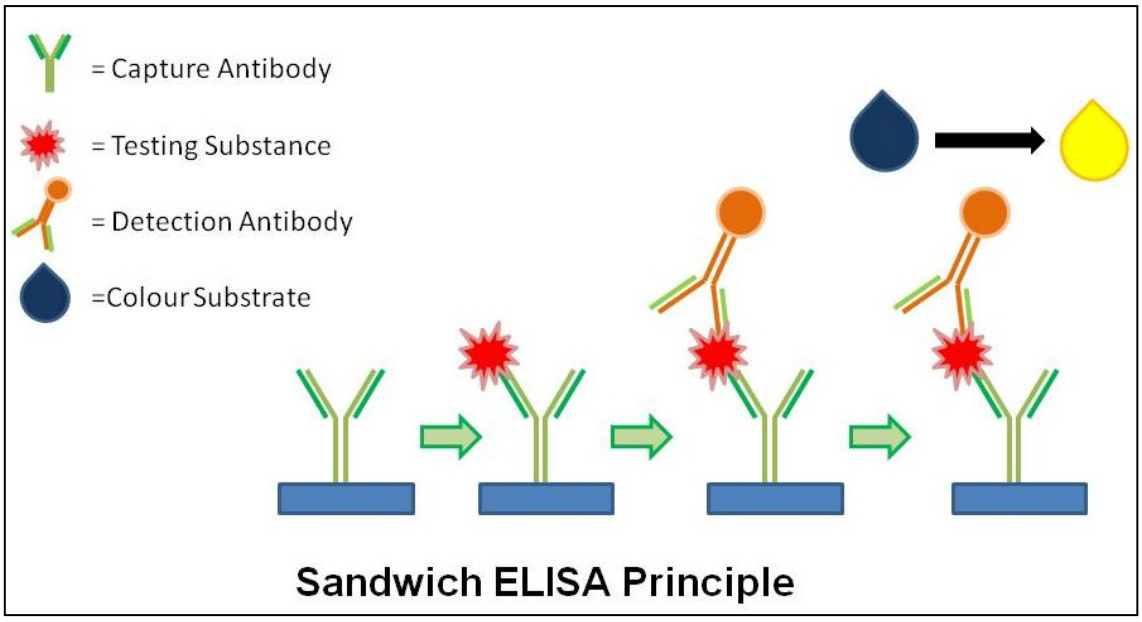


**Figure 6** **The principle of the sandwich ELISA**

### 6.10.1.2 The competitive ELISA

The competitive ELISA is based on the relation between the testing substance and a substance, preconjugated with an enzyme. As in the sandwich ELISA the capture antibody is coated on the ground of the test well plates. The principle in this test is that the coated antibodies are limited in the wells. The testing substance and the preconjugated substance compete against each other for the binding spots of the antibodies. After a binding time the not bound substances are removed, leaving a defined amount of captured substances, which is equivalent for the relation between testing and preconjugated substances. Now the colour substrate is added which changes its colour equal to the bound preconjugated substance. The process is stopped by a stop solution and the extinction is measured. In this test, differential to the sandwich ELISA, the wells with a low rate of the substance of interest in the highest level of colour change. (Figure 7) For concentration calculation the concentration will be determined like in the sandwich ELISA by a standard curve. (128, 130)

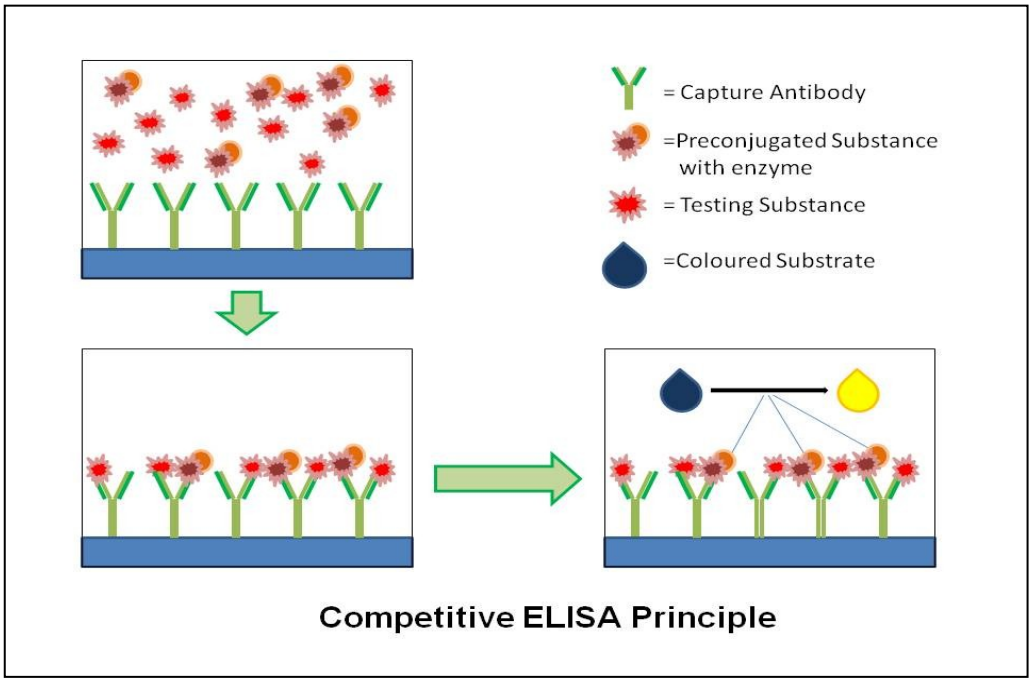


**Figure 7** **The principle of the competitive ELISA**

## 6.11 Measurement and data processing

All testing units have been measured at least in duplicates. As a final step, the optical density of the developed 96 well plates of all tests have been measured with Spark® multimode microplate reader at the tests specified wavelength of 450 nm, only the serotonin test kit needed 405 nm as testing wavelength. To eliminate optical effects of the plate itself or other undesired impact factors, a measurement at 555 nm has been subtracted from the results. During the further procedure a regression curve had been made out of the test standard values and the measured values of interest have been converted from optical density into substance concentrations. The duplicated values have been averaged for the statistical data processing.

## 6.12 Laboratory equipment

- Labculture® Class II (Low Noise) Biosafety Cabinet
- Beckman Coulter Allegra$^{TM}$ X-12R Centrifuge
- Liebherr Mediline Kühlschrank -20°C
- Dynex MRW Plate Washer
- Cytoperm$^{TM}$ 2 $CO_2$ Gassed Incubator
- Heidolph Titramax 1000 Schüttel- & Mischgerät
- Sysmex KX-21N automated hematology analyzer
- Spark® multimode microplate reader from TECAN

## 6.13 Statistics

The statistic evaluation was made with SPSS (SPSS Inc., Chicago, USA) and GraphPad Prism 5 (GraphPad Software Inc., California, USA). For Sample size calculation an online sample size calcualtor (http://powerandsamplesize.com/Calculators/Compare-2-Means/2-Sample-Equality http://homepage.stat.uiowa.edu/~rlenth/Power/) was used. Based on results of former studies in the laboratory the sample size of 5 donors have been defined as adequate to determine a difference with a probability of 80% (alpha=0,05) in the test groups implementing a one-way ANOVA or Kruskal Wallis test. As post-hoc test for

multiple testing Tukey correction or in case that the results are not normally distributed Dunn´s multiple comparison test is used. Shapiro-Wilk normality test is used for testing the results for Gaussian distribution. As statistical significant, P-values below 0.05, as statistically very significant P-values below 0.01 and as statistically extremely significant, P-values below 0.001 were defined.

The data, generated from the microplate reader have been evaluated after instructions of the test kit guidance before statistical evaluation was made.

# 7 Results

## 7.1 Cell viability assay

### 7.1.1 Methylene blue manipulates the toxicity assay

After treatment of peripheral blood mononuclear cells with serial diluted methylene blue, the following effect has been determined: Methylene blue in high concentrations provided due to its strong self-colouring no measurable results. To eliminate this problem, a serial dilution of methylene blue was made to subtract the blank value from the treated cell value, but the strong self-colouring did not allow a precise measurement. Just attenuations under 1:64 served doubtful results of viability in the field of 40%. Results of cells treated with diluted Proveblue under a concentration of 0,61 mg/L depicts definitely no toxicity of the methylene blue any more. This draws the conclusion that at least a part of PBMCs treated with Proveblue after dilution of more than 1:64 are still alive and useful for further studies. (Figure 8) Consulting recommended concentrations of Proveblue in clinical use, with the injection based way of treatment in mind that implies a direct contact of methylene blue with blood cells without any special notes for care. No further toxicity assay was made for methylene blue.

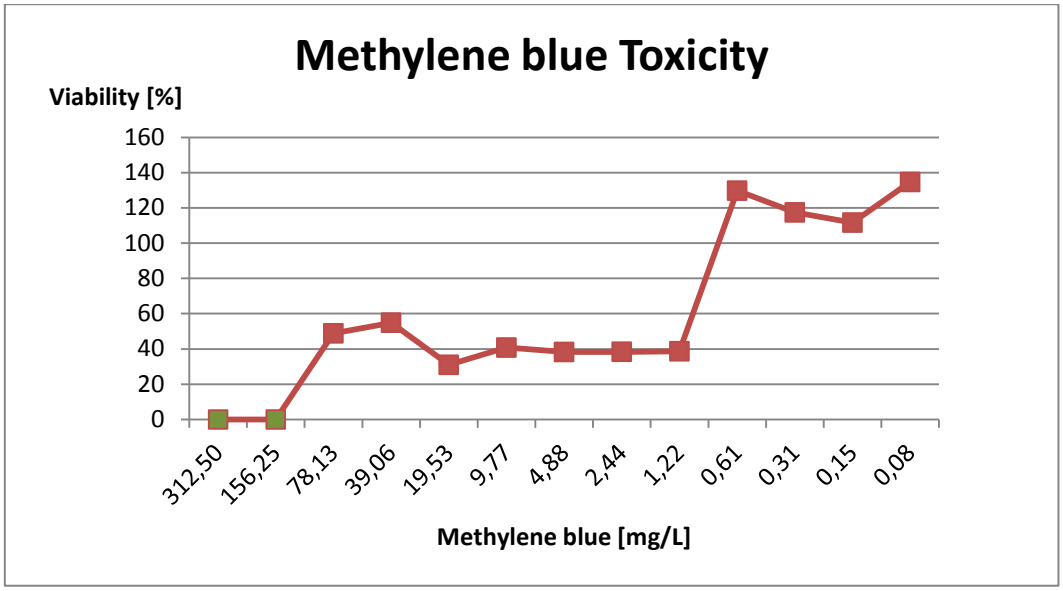


**Figure 8** **Methylene blue manipulates the toxicity assay.**
The Figure depicts the toxic effect from methylene blue in different dilution steps on PBMCs in a concentration of 3x10^6 cells/ml. The result is disputable. Because of the strong self-colouring from methylene blue, values with methylene blue concentrationshigher than 78,13 mg/L were not measurable. It is not possible under this impact factor to make a statement about the toxicity of methylene blue, but it concludes that a part of PBMCs treated with diluted Proveblue [methylene blue (5mg/ml)] with concentrations under 78,13 mg/L are still alive and suitable for our studies.

## 7.1.2 Nicotine effects a viability limitation

The toxicity assay EZ4U revealed that the addition of nicotine to a peripheral blood mononuclear cells solution has a potential, concentration dependent toxic effect on the viability of PBMCs. The viability rises by the decrease of the nicotine concentration and reaches nearly 100 % after cell treatment with a nicotine concentration of $1.25x10^{-5}$ mg/L. (Figure 9) The approximately constant effect of nicotine in concentrations higher than $1,25x10^{-3}$ hypothesizes a proliferation inhibiting impact.

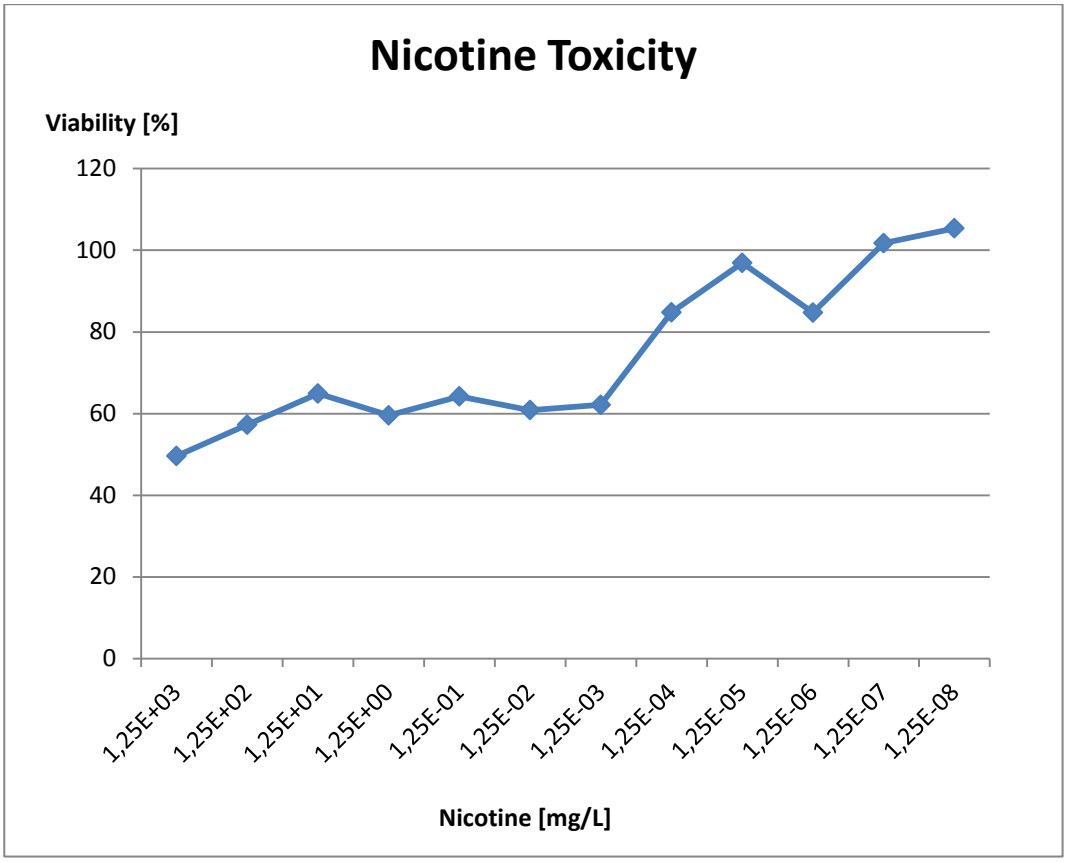


**Figure 9** **Nicotine effects a viability limitation.**
The Graph shows the effect from nicotine (50mg/ml) after a rash of dilutions on PBMCs in a concentration of 3x10^6 cells/ml. A concentration dependent trend of toxicity is distinguishable. Especially the impact of nicotine in concentrations higher than $1{,}25x10^{-3}$ show a nearly steady effect on the cells resulting with a viability about 60%.

# 7.2 ELISA detection

## 7.2.1 Methylene blue reduces S100A8/A9 secretion

PBMCs reveal a significant reduced secretion pattern of S100A8/A9 after treatment with methylene blue. Compared to the untreated control, treatment with concentrations of 6,25 mg/L and 25 mg/L show significant reduced S100A8/A9 concentrations after an incubation time of 24 hours. Treatment concentration of 1,56 mg/L reveals no significance. As control untreated PBMCs from the same donors were processed the same way. (Figure 10) On the other side, no significant effect concerning the S100A8/A9 release by PBMCs occurs after treatment with nicotine in two different concentrations (50 ng/ml; 5000 ng/ml). (Figure 11)

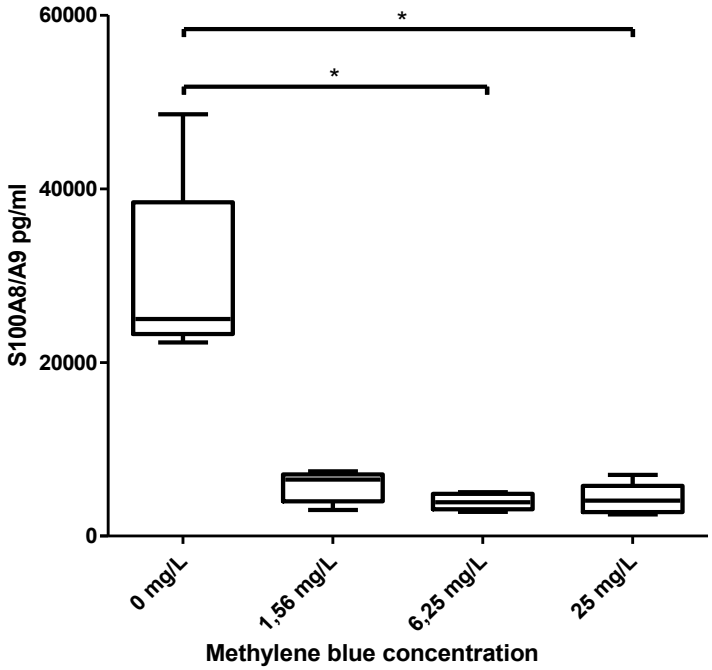


**Figure 10** **Methylene blue reduces S100A8/A9 secretion**
The Figure shows the downregulation of the secretion of the antimicrobial peptide S100A8/A9 (Calprotectin) from PBMCs by treatment with methylene blue for 24 hours. Treatment with a concentration of 6,25mg/l and 25mg/l methylene blue show a significant reduction of S100A8/A9 secretion compared to untreated PBMCs with 0mg/l methylene blue. (n=5) (p=0,0068)

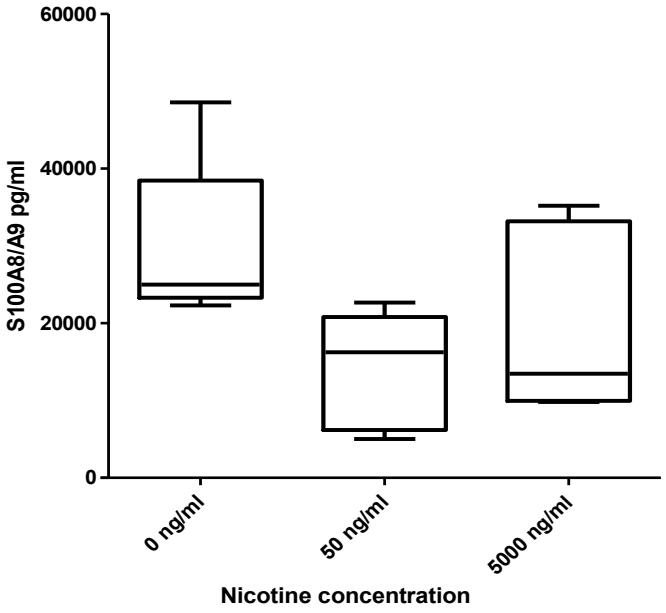


**Figure 11** **Nicotine shows no significant effect on S100A8/A9 secretion**
After Treatment of $25x10^6$ PBMCs/ml with Nicotine for 24 hours, no effect on the S100A8/A9 release has been detected. Untreated cells undergoing the same processes act as control. (n=5)

### 7.2.2 Methylene blue and Nicotine reveal no impact on LL37 and alpha defensin-1 secretion

No significant effect by the treatment of PBMCs with methylene blue or nicotine was discovered on the LL37 and the alpha defensin-1 secretion. (Figure 13;Figure 14;Figure 15) After 24 hours incubation with three different methylene blue concentrations (1,56 mg/L; 6,25 mg/L; 25 mg/L) or two different nicotine concentrations (50 ng/ml; 5000ng/ml) no influence on the secretion pattern occurs compared to untreated, incubated PBMCs as control. Only the LL37 secretion after methylene blue shows a spreading impact. (Figure 12)

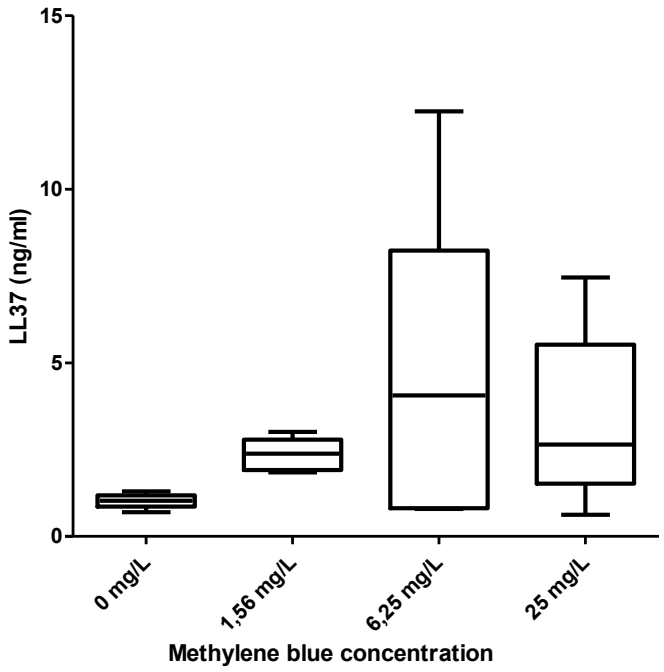


**Figure 12** **Methylene blue reveals no significant effect on the secretion of LL37**
The **t**reatment of PBMCs with three different concentrations of methylene blue shows no significant effect on the secretion of LL37 (Cathelicidine). Untreated PBMCs undergoing the same processes acts as a control. (n=5) A potential trend could be possible, but due to the variance of results and the small testing group, no significance is detectable**. (n=5)**

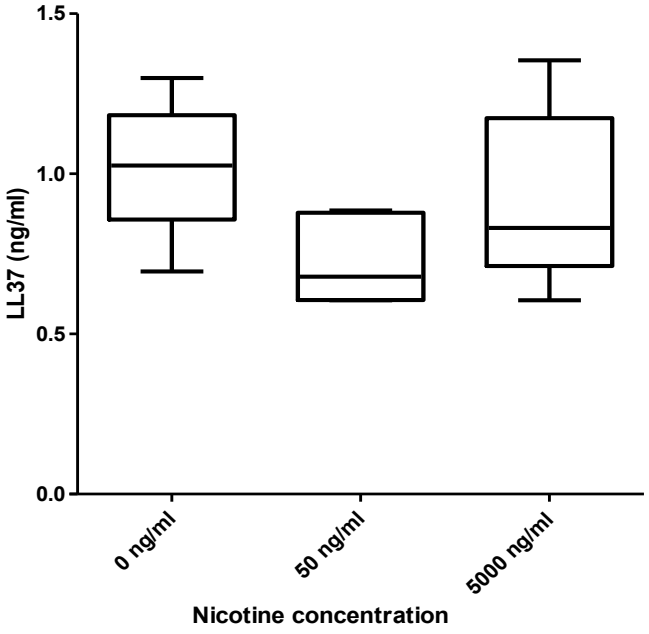


**Figure 13** **Nicotine has no relevant impact on the reveal of LL37**
PBMCs show no significant change in their LL37 secretion behave after treatment with nicotine in two different concentrations. PBMCs without treatment substance act as control. (n=5)

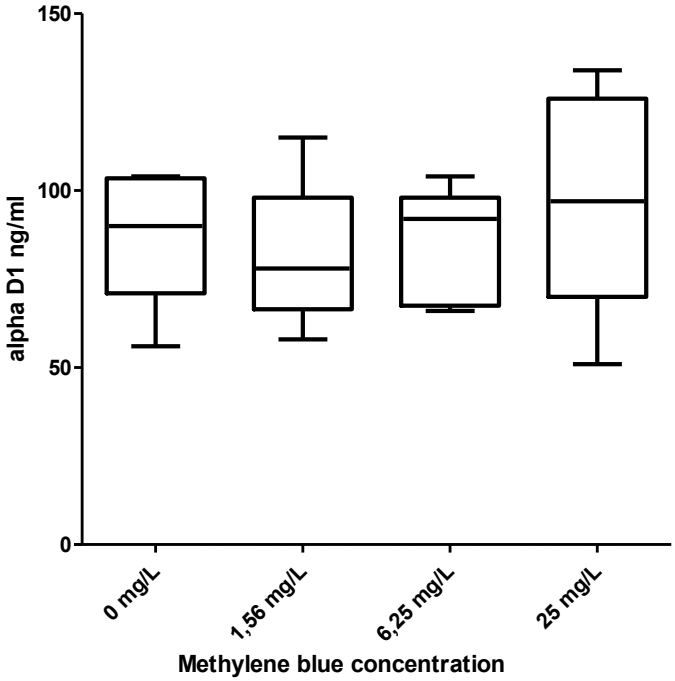


**Figure 14** **Methylene blue shows no significant impact on the release of alpha defensin-1**
The treatment of PBMCs with three different concentrations of methylene blue results no significant effect on the secretion of alpha defensin 1. As control untreated PBMCs have been processed the same way. (n=5)

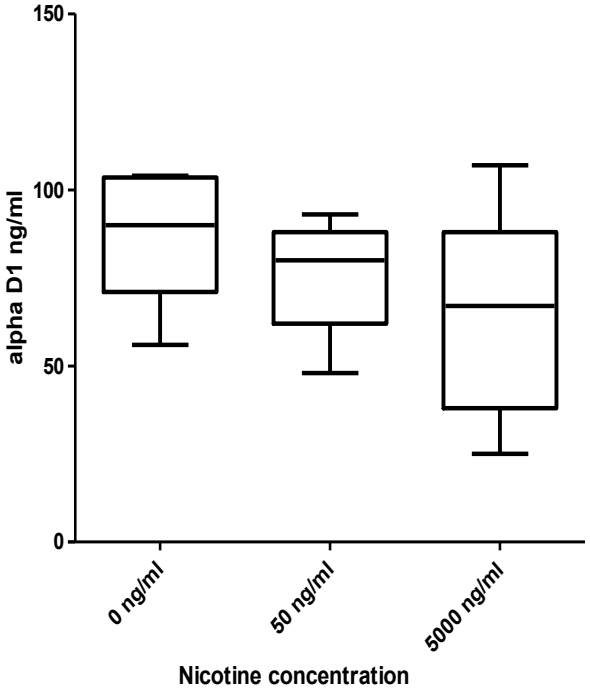


**Figure 15** **Nicotine shows no detectable effect on the secretion of alpha defensin-1 by PBMCs**

After treatment of 25x10$^6$ PBMCs/ml with nicotine in different concentrations for 24 hours, no significant impact has been detected. Untreated PBMCs undergoing the same study-processes act as control. (n=5)

### 7.2.3 Methylene blue and Nicotine influence Serotonin secretion in special ways

After treatment of 25x10$^6$ PBMCs/ml for 24 hours with methylene blue, a highly significant difference, concerning the Serotonin secretion, between the treatment with 1,56 mg/L and 25 mg/L was detected. On the one side treatment with the lowest methylene blue concentration leads to high Serotonin secretion up to 650 nmol/L, on the other side high concentrated treatment with 25 mg methylene blue/ml results in nearly not detectable serotonin concentrations. The untreated control as well as the with 6,25 mg methylene blue /ml treated cells depict a nearly similar low result under 100 nmol serotonin /L, with no significant concentration differences. (Figure 16)

Nicotine in treating concentrations of 50 ng/ml and 5000 ng/ml reveal no significant impact on the secretion of serotonin by PBMCs after 24 hours incubation time. The same control as in the methylene blue treatment analysis was used. In the practical procedure a donor dependent pattern was recognisable, resulting in the visible trend in the figure. (Figure 17) Specific donor cells revealed higher results after treatment

with nicotine for 24 hours then the others. This was noticeable in the ELISA-well plate as well as in the Graph.

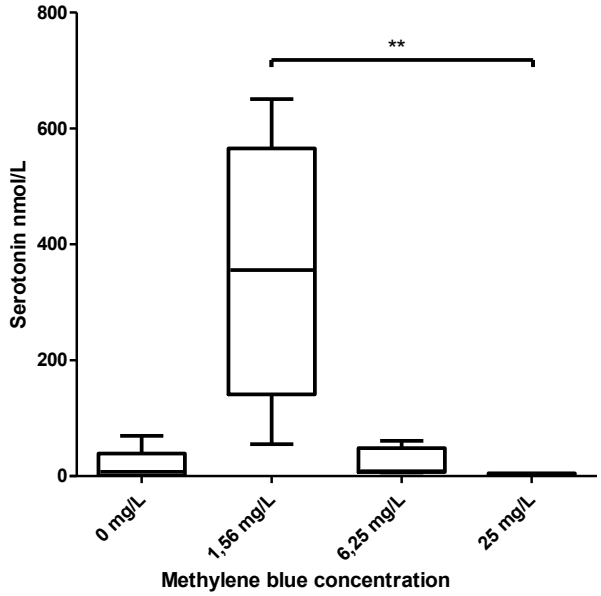


**Figure 16** **Methylene blue reveals concentration dependent impact on the serotonin release**
24 hours of treatment with methylene blue in three different concentrations depict a concentration dependent influence on the Serotonin secretion of PBMCs. Treatment with 6.25 mg/L seems to result similar effects like cells without any treatment. Whereas a concentration of 1.56 mg/L reveals a significantly higher serotonin release, up to 650 nmol/L, than a methylene blue concentration of 25 mg/L with resulting Serotonin concentrations in a nearly not detectable concentration. PBMCs without any special treatment act as control. (n=5)

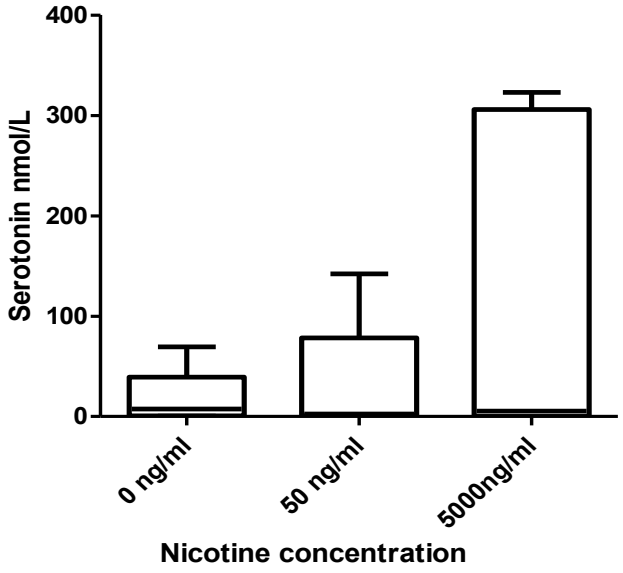


**Figure 17** **Nicotine treatment reveals a concentration dependent trend of serotonin release without any significance** The effect of nicotine in two different concentrations on the secretion of Serotonin by PBMCs after 24 hours results no significant impact. Nonetheless a donor dependent trend attracted attention in practical procedure and is also noticeable in the resulting Graph. (n=5) Untreated PBMCs undergoing the same study-processes are used as control.

### 7.2.4 Treating Substances show no impact on the Adrenaline secretion pattern

The separated PBMCs of 5 donors, in a concentration of $25x10^6$ PBMCs/ml, have been incubated for 24 hours with methylene blue in three different concentration (1,56 mg/L; 6,25 mg/L; 25 mg/L) or nicotine in two different concentrations (50 ng/ml; 5000 ng/ml). This treatment revealed no significant influence on the secretion of adrenaline, detected via ELISA testing. Incubated PBMCs without any special treatment acted as control.

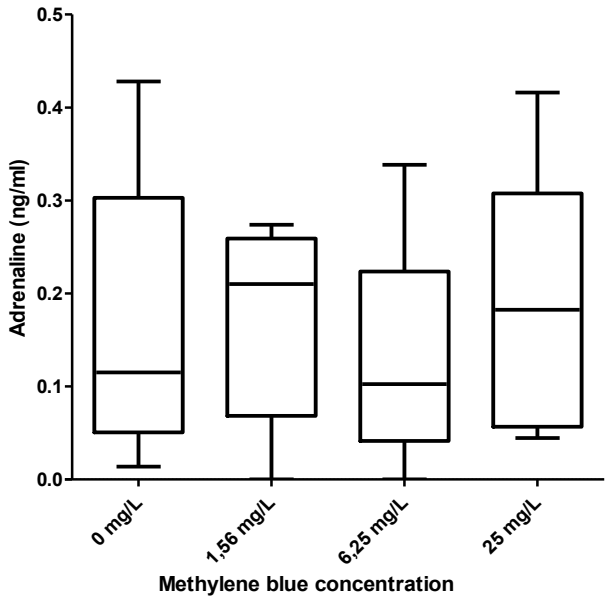


**Figure 18** **Treatment with methylene blue shows no significant influence on the secretion of adrenaline**
Methylene blue treatment of PBMCs in the concentration $25x10^6$ cells/ml shows any influence on the release of adrenaline. As a control PBMCs without any special treatment have been chosen. (n=5)

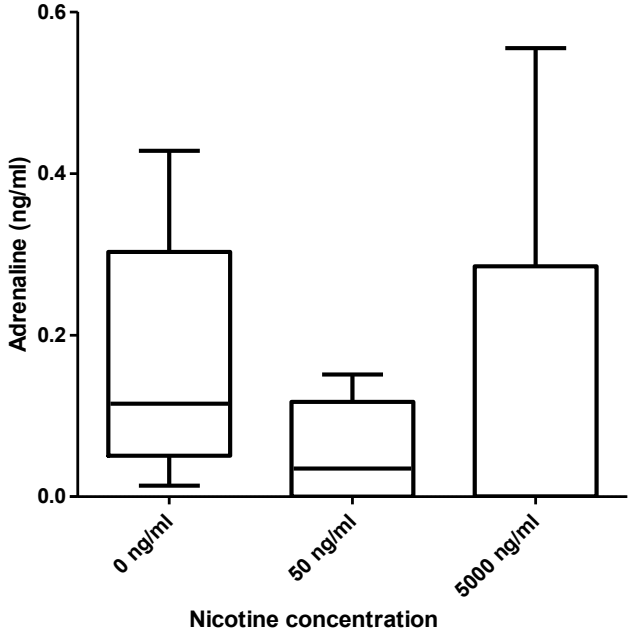


**Figure 19** **Nicotine reveals no significant impact on the adrenaline secretion**
After addition of Nicotine in two different concentrations and incubation for 24 hours, no significant change in the adrenaline secretion pattern of PBMCs has been detected. (n=5) As control untreated PBMCs processed the same way are used.

### 7.2.5 Nicotine increases the Dopamine secretion of PBMCS, whereas methylene blue only presents a potential trend

Nicotine affects an increase in dopamine secretion of PBMCs. After treatment with two different nicotine concentrations 50 ng/ml result a very significant and 5000 ng/ml an extremely significant difference compared to the untreated control. After 24 hours of incubation the resulting concentrations have been detected by ELISA testing. (Figure 21)

The chosen concentrations of methylene blue showed no significant influence on the dopamine release by PBMCs, just a concentration dependent trend involving a growing mean variation is recognisable after 24 hours of incubation. As control untreated, incubated PBMCs in the same concentration ($25x10^6$ cells/ml) have been used. (Figure 20)

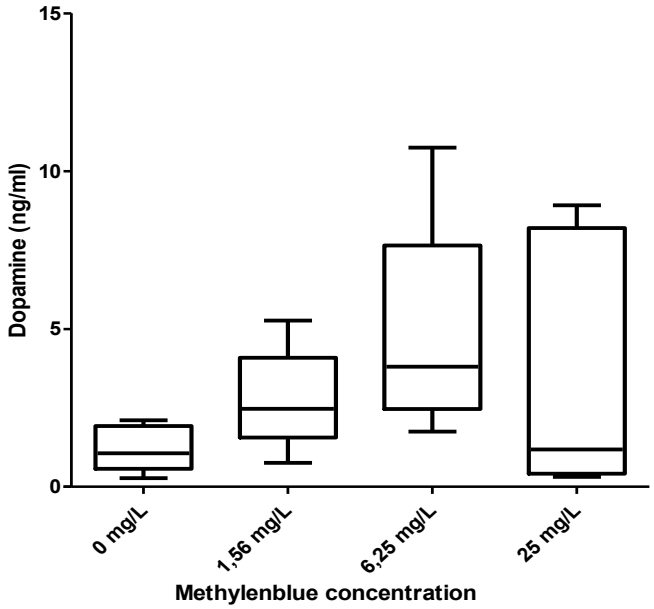


**Figure 20** **Methylene blue depicts a promising trend without any significance regarding dopamine secretion**
The treatment of PBMCs with methylene blue for 24 hours shows no significant effect on the Dopamine secretion. Though, a visible trend is recognizable, that increasing methylene blue concentrations affect increasing Dopamine release. As control also processed PBMCs without any treatment were used. (n=5)

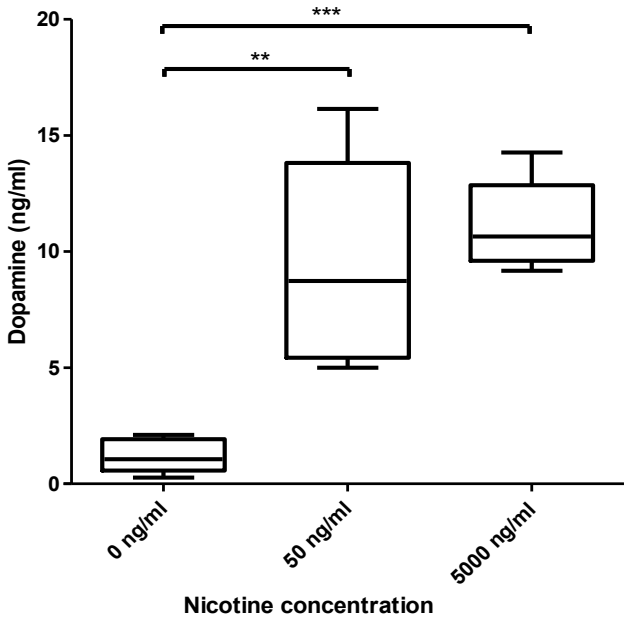


**Figure 21** **Nicotine leads PBMCs to boost their dopamine secretion significantly**
A significantly higher concentration of Dopamine was detected after treatment of $25x10^6$ PBMCs/ml with nicotine in two different concentrations for 24 hours. (n=5) Untreated PBMCs undergoing the same processes are used as control. The secretion of Dopamine is very significant (50ng/ml nicotine) and extremely significant (5000ng/ml nicotine) increased compared to the untreated control.

### 7.2.6 Nicotine inhibits PBMCs noradrenaline secretion

Nicotine results a decrease in PBMCs noradrenaline secretion after incubation of 24 hours. The amount of $25x10^6$ PBMCs/ml was treated with two different concentrations of nicotine (50 ng/ml; 5000ng/ml). Compared to untreated, incubated PBMCs that act as control, a significant decrease was detectable after treatment with 50 ng nicotine/ml as well as a highly significant decrease of the noradrenaline secretion after treatment with 5000 ng/ml. (Figure 23)

In case of methylene blue influencing the noradrenaline secretion of PBMCs, no significant result was ascertainable. Wide spreading of the results from the with three different methylene blue concentrations (1,56 mg/L; 6,25 mg/L; 25 mg/L) treated groups is conspicuous compared to the untreated control. In contrast to the untreated control, the treated groups reveal a decreasing trend on the noradrenaline secretion after 24 hours incubation. (Figure 22)

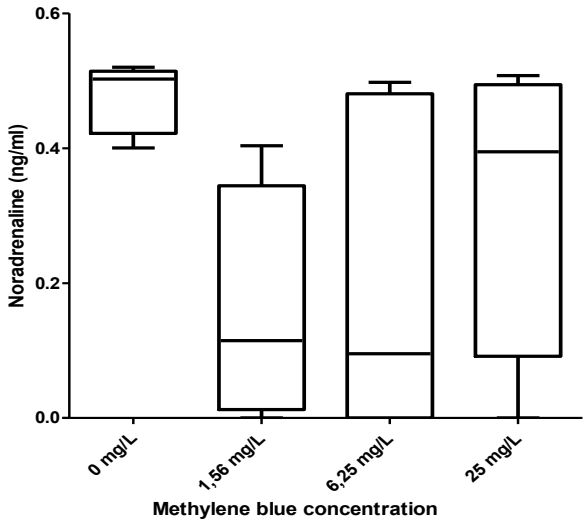


**Figure 22** **Methylene blue reveals no significant impact on noradrenaline secretion**
After treatment with methylene blue no significant influence on the secretion pattern of PBMCs in relation to noradrenaline was detected. Nonetheless, the result highlights a potential decreasing in context with the treatment. This effect occurs donor dependent resulting in a huge spreading of the measured datas. A concentration of $25x10^6$ PBMCs/ml with a 24 hours incubation time leads to the results. As control PBMCs without any special treatment were used. (n=5)

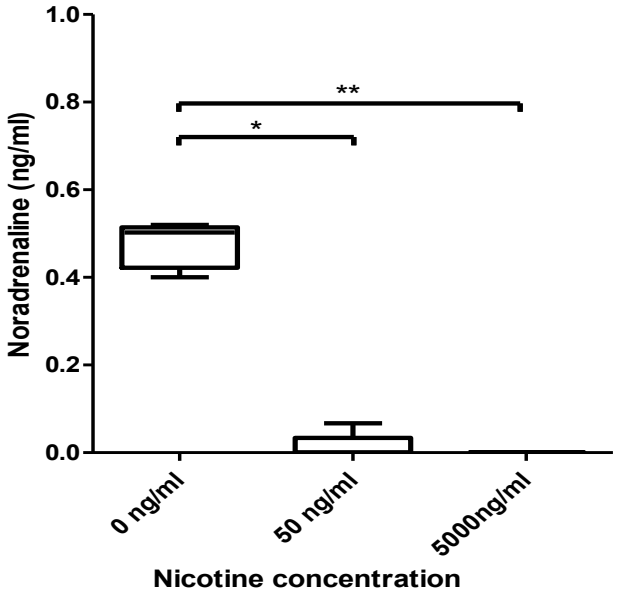


**Figure 23** **Nicotine inhibits PBMCs noradrenaline secretion**
PBMCs present a decreasing Noradrenaline secretion after treatment with nicotine for 24 hours. (n=5) Untreated PBMCs undergoing the same processes act as control. The secretion from Noradrenalin is significant (50ng/ml nicotine) and very significant (5000ng/ml nicotine) decreased compared to the untreated control (0ng/ml nicotine).

# 8 Discussion

The basic idea of this study was to analyse the effect of methylene blue and nicotine on the secretion of antimicrobial peptides LL37, S100A8/A9 (calprotectin) and defensin alpha-1 and the release of the neurotransmitter noradrenaline, dopamine, adrenaline and serotonin from peripheral blood mononuclear cells. Preceding studies published in 2016 by Kasiri et al., demonstrated that stressed peripheral blood mononuclear cells secrete antimicrobial peptides. Therefore, we wanted to observe if methylene blue effects this secretion. Moreover, methylene blue may also stress these cells and therefore trigger the production and secretion of AMPs and neurotransmitters.

The study revealed that, methylene blue manipulates peripheral blood mononuclear cells in their nature as S100A8/A9 (calprotectin) releasing cells. We could show that PBMCs secrete a distinct increased level of S100A8/A9 when cultured without the presence of methylene blue. This discharged substance is a well known inflammation factor with impact on other inflammation factors and has zinc dependant impact on important functions like wound healing, angiogenesis, tissue destruction and embryonic development. (19, 92, 93) PBMCs are a heterogeneous cell population and from all cell types of PBMCs calprotectin is found in monocytes, but not in lymphocytes. (64) Treatment with methylene blue leads monocytes to lower their calprotectin secretion, by inhibiting a novel pathway. This pathway usually activates the mitogen-activated protein (MAP) kinase cascade and requires an intact microtubule system. (131, 132) Calprotectin has concentrations dependent inhibiting or killing effects on the proliferation of yeasts and bacteria. The antimicrobial function is absent in blood agar. The assumptions was made that this effect may result from lytic enzymes released from leucocytes. (91) With this in mind a physiological meaning of the result for treatment in patients is doubtful. Assuming this lytic effect is not relevant the calprotectin increasing effect of methylene blue causes a decreased direct protective effect against pathogens and missing chemotactic and cell growth inhibiting function but also reduces inflammation response in case of infections with possibly a positive outcome on patient´s wellbeing. (133, 134)

The present results allow speculation that methylene blue may have a conflicting effect on the serotonin secretion. Low concentrations might stimulate the serotonin production from peripheral blood mononuclear cells whereas a higher concentration of methylene blue does not lead to an increased release. PBMCs without MB contact do not secrete serotonin neither. Methylene blue inhibits monoamine oxidase, resulting in a triggering function concerning serotonin toxicity. (4) An additional serotonin-releasing effect of methylene blue on PBMCs promotes a high concentration of serotonin in the organism. This might identify another reason for contraindication in treatment of patients with depression. Human lymphocytes express a serotonin reuptake transporter (SERT). T-lymphocytes are further able to synthesize serotonin.(27, 135) Relating to the current results, methylene blue in low concentrations either stimulates the production of serotonin inside the lymphocytes by possibly affecting the TPH-1 enzyme intracellular or it may inhibit SERT of lymphocytes resulting in an increasing extracellular concentration. Assuming that assumption is true, the question arises which concentration of methylene blue results in the highest influence on the serotonin secretion, and why do lower concentrations have more effect than higher. To detect this possible impact an analysis at lower concentrations including a higher number of different concentration treatments is needed. The spreading of the values after addition of low methylene blue concentration might cause different reasons, like habit depending conditioning from donors or congenital differences in cell behaviour.

Methylene blue also may influence the secretion of dopamine. We could detect a dose dependent alteration of dopamine concentration in the supernatant of the cultured and stimulated PBMCs after 24 hours. A higher methylene blue concentration led to a higher dopamine concentration. Due to high donor variability especially in the high dose group, these data were not significant and only a trend was detected. Maybe a significant effect can be observed at higher methylene blue concentrations.

After consuming one cigarette, a nicotine concentration of about 40 ng/ml can be determined in the serum. Peripheral blood mononuclear cells increase their dopamine release after treatment with nicotine. This effect might support the known function of nicotine by releasing dopamine from the adrenal glands.(119) Dopamine is produced by lymphocytes as Bergquist et al. revealed in 1994. (20) In 2004 Ferrari

et al. discovered that Protein kinase C (PKC) activators induce the expression of the Tryptophan Hydroxylase (TH) mRNA. (136) This suggests a possible PKC activating function of nicotine to support the TH production and in a result the synthesis of dopamine. Dopamine in conjunction with T-cells performs autocrine as well as paracrine functions. It is also important for antigen-specific interactions between T-cells and dendritic cells and in some autoimmune diseases and neurological/psychiatric diseases a disturbed T-cell-dopamine-metabolism is detected. (137) However, the secretion of dopamine by blood cells appears in the whole body whereas the dopamine produced by the adrenal glands has to cover the distance between the producing organ and its targets. The direct secretion near the translating cells may cause an earlier effect. This could be seen as a warm-up for the binding cells, before the intensive, high concentrations of the released neurotransmitters reaching the target binding through the receptors on the cell membrane. In this study the nicotine effect has been detected after 24 hours. 24 hours after the adding the nicotine to the PBMC-cell suspension an increased dopamine concentration has been measured. The half life of neurotransmitter is just a few hours. This fact draws the conclusion that the stimulating effect of nicotine on analysed blood cells for a long time causes a secretion over many hours. This accounts interest in the short-term influence of nicotine on peripheral blood mononuclear cells.

The inhibiting effect on the noradrenaline secretion of PBMCs shows again the subtle distinctions in action between the neurotransmitters. Paradoxically to the dopamine increasing effect of nicotine, the secretion of noradrenaline is decreased. No similar effect is reported in context with T-cells neurotransmitter secreting functions. Considering the results, nicotine seems to have an inhibiting effect on dopamine-ß-hydroxylase, the enzyme that builds noradrenaline from dopamine. Though, nicotine induces noradrenaline secretion in the chromaffine cells of the adrenal glands so the relevance of noradrenaline release by blood cells might be minimal. (119)

In this study setting, nicotine did not reveal any significant effect on the secretion of serotonin from peripheral blood mononuclear cells. Just an increasing trend can be deduced. During the ELISA procedure, a donor dependent pattern was noticeable. There were two donors that did not show any reaction on nicotine. In contrast, the three other donors secreted more serotonin in a concentration dependent manner.

Thus, the assumption of habit depending causation suggests itself. The smoking habits of the donors are missing because of discrete practice and because it is not queried at the blood donation. The revealed results demand an evaluation in this direction. It is from interest, if the three responders are smokers and are sensitized for nicotine or if they are non-smoker and the threshold of the triggering of serotonin production is lower compared to smokers. The human body causes tolerance after consumption of nicotine over a long period, so a direct effect on the blood cells by nicotine would be a syllogism.(123)

In this study the effect after 24 hours of nicotine presence on PBMCs has been analysed. A possible long-term effect, as well as detailed concentration dependent effect needs further research.

One important point to recognise is the fact that the blood brain barrier does not allow neurotransmitters to pass, so the bearing of neurotransmitters built by blood cells on the central nervous system has to be seen critical. Just a central impact as consequence of peripheral neurotransmitter activity is possible.

In preliminary experiments, the toxicity of the tested substances, methylene blue and nicotine, on peripheral blood mononuclear cells was evaluated by EZ4U test. The toxicity of methylene blue was not able to be detected because of the intensive colour from methylene blue. So the link for influence of possible apoptosis is missing. Nicotine on the other side showed definitely toxicity on the PBMCs. The way of this toxicity is not determined. It could be an apoptotic but also a necrotic effect. As already shown in several studies by the research group of Univ.-Prof. Dr. Hendrik J. Ankersmit, apoptotic PBMCs produce a secretome with enormous different functions. Starting with vasodilatation, angiogenesis, immunomodulation, cytoprotection and induction of neutrophic factors over M1-M2 polarization, platelet inhibition, reepithelialization and – important and decisive for this study - the antimicrobial function by the release of antimicrobial peptides. So a possible apoptotic effect of nicotine or methylene blue would support the human body in these different important areas. Nevertheless, we do not recommend the consumption of nicotine as the negative effects especially from smoking are severe.

A limitation in this study is the chosen amount of blood donors. Especially in case of nicotine the impact of the donor habits might influence the results. The long-term

nicotine consumption affects the blood cells and also results in a deadening effect. To avoid the measurable blackout by this factor, beside the natural concentration of nicotine after one cigarette in blood, a hundredfold concentration has been chosen.

One important characteristic of neurotransmitters is their short half-life. After the addition of the test substances a possible immediate release of neurotransmitters, especially in the focus on the in vesicles saved neurotransmitters in B-cells, was not able to be detected in this study setting. To analyse the direct influence an analyses with a shorter incubation time is suggested.

**Conclusion:**

The present study revealed the increasing effect of methylene blue on the secretion of S100A8/A9 (calprotectin) as well as the concentration dependent influence on the release of serotonin by peripheral blood mononuclear cells. Further the increasing impact of nicotine on the secretion of dopamine and the decreasing effect on the secretion of noradrenaline by peripheral blood mononuclear cells has been detected. Nicotine also confessed a promising trend on boosting the secretion of serotonin which may be a further way to explain the feeling of rewarding of smokers

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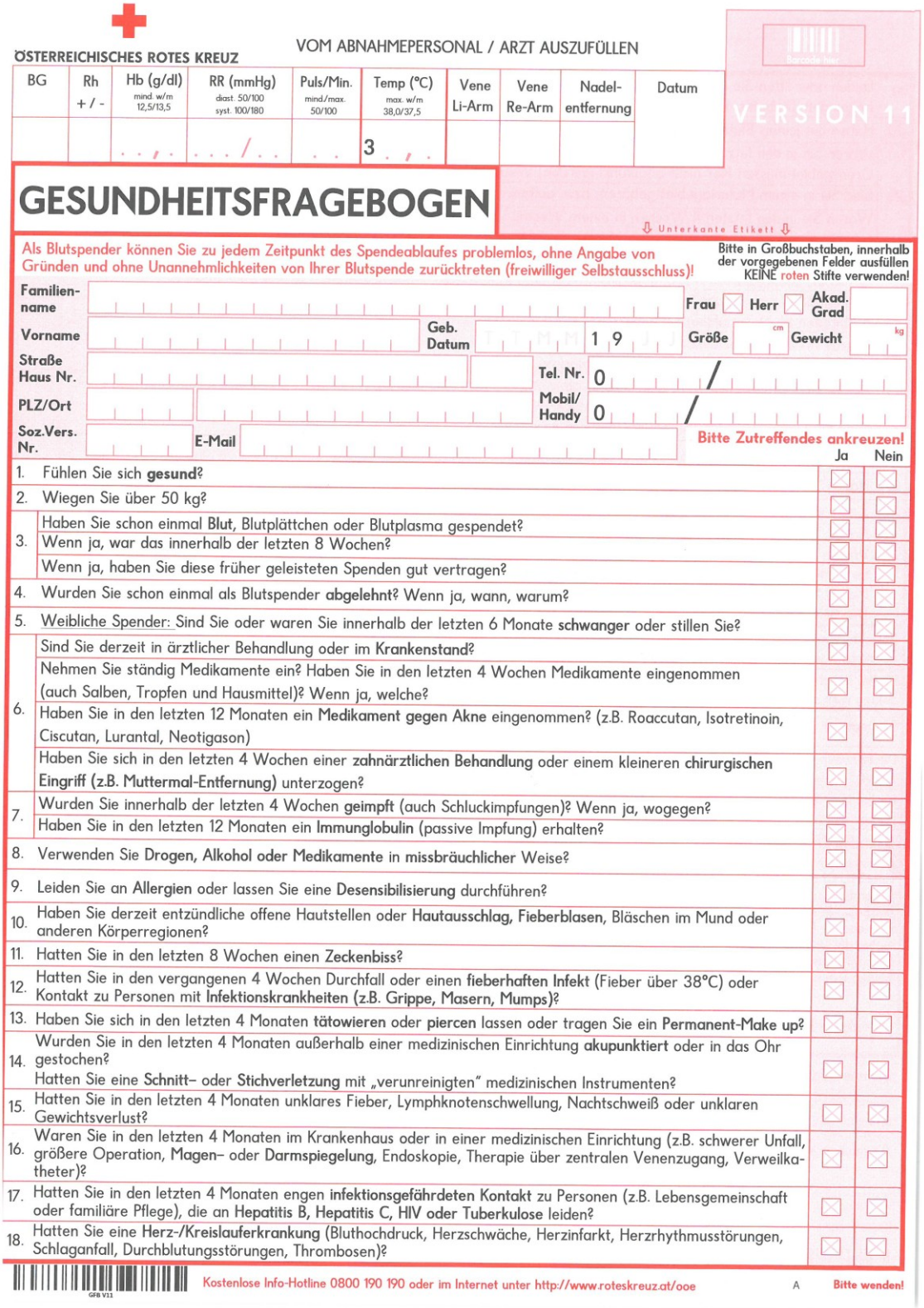
# 9 Attachment

## 9.1 List of Figures

# 9.2 Red-Cross Health-Form



ÖSTERREICHISCHES ROTES KREUZ

VOM ABNAHMEPERSONAL / ARZT AUSZUFÜLLEN

| BG | Rh +/- | Hb (g/dl) mind. w/m 12,5/13,5 | RR (mmHg) diast. 50/100 syst. 100/180 | Puls/Min. mind./max. 50/100 | Temp (°C) max. w/m 38,0/37,5 | Vene Li-Arm | Vene Re-Arm | Nadel-entfernung | Datum |
|---|---|---|---|---|---|---|---|---|---|
| | | . . , . | . . . / . . | . . | 3 . , . | | | | |

VERSION 11

## GESUNDHEITSFRAGEBOGEN

⇩ Unterkante Etikett ⇩

Als Blutspender können Sie zu jedem Zeitpunkt des Spendeablaufes problemlos, ohne Angabe von Gründen und ohne Unannehmlichkeiten von Ihrer Blutspende zurücktreten (freiwilliger Selbstausschluss)!

Bitte in Großbuchstaben, innerhalb der vorgegebenen Felder ausfüllen KEINE roten Stifte verwenden!

Familienname | Frau ☒ Herr ☒ | Akad. Grad

Vorname | Geb. Datum T T M M 1 9 J J | Größe cm | Gewicht kg

Straße Haus Nr. | Tel. Nr. 0 /

PLZ/Ort | Mobil/Handy 0 /

Soz.Vers. Nr. | E-Mail

Bitte Zutreffendes ankreuzen!

| | | Ja | Nein |
|---|---|---|---|
| 1. | Fühlen Sie sich **gesund**? | ☒ | ☒ |
| 2. | Wiegen Sie über 50 kg? | ☒ | ☒ |
| 3. | Haben Sie schon einmal **Blut**, Blutplättchen oder Blutplasma gespendet? | ☒ | ☒ |
| | Wenn ja, war das innerhalb der letzten 8 Wochen? | ☒ | ☒ |
| | Wenn ja, haben Sie diese früher geleisteten Spenden gut vertragen? | ☒ | ☒ |
| 4. | Wurden Sie schon einmal als Blutspender **abgelehnt**? Wenn ja, wann, warum? | ☒ | ☒ |
| 5. | Weibliche Spender: Sind Sie oder waren Sie innerhalb der letzten 6 Monate **schwanger** oder stillen Sie? | ☒ | ☒ |
| 6. | Sind Sie derzeit in ärztlicher Behandlung oder im **Krankenstand**? | ☒ | ☒ |
| | Nehmen Sie ständig Medikamente ein? Haben Sie in den letzten 4 Wochen Medikamente eingenommen (auch Salben, Tropfen und Hausmittel)? Wenn ja, welche? | ☒ | ☒ |
| | Haben Sie in den letzten 12 Monaten ein **Medikament gegen Akne** eingenommen? (z.B. Roaccutan, Isotretinoin, Ciscutan, Lurantal, Neotigason) | ☒ | ☒ |
| | Haben Sie sich in den letzten 4 Wochen einer **zahnärztlichen Behandlung** oder einem kleineren **chirurgischen Eingriff (z.B. Muttermal-Entfernung)** unterzogen? | ☒ | ☒ |
| 7. | Wurden Sie innerhalb der letzten 4 Wochen **geimpft** (auch Schluckimpfungen)? Wenn ja, wogegen? | ☒ | ☒ |
| | Haben Sie in den letzten 12 Monaten ein **Immunglobulin** (passive Impfung) erhalten? | ☒ | ☒ |
| 8. | Verwenden Sie **Drogen, Alkohol oder Medikamente** in **missbräuchlicher** Weise? | ☒ | ☒ |
| 9. | Leiden Sie an **Allergien** oder lassen Sie eine **Desensibilisierung** durchführen? | ☒ | ☒ |
| 10. | Haben Sie derzeit entzündliche offene Hautstellen oder **Hautausschlag, Fieberblasen**, Bläschen im Mund oder anderen Körperregionen? | ☒ | ☒ |
| 11. | Hatten Sie in den letzten 8 Wochen einen **Zeckenbiss**? | ☒ | ☒ |
| 12. | Hatten Sie in den vergangenen 4 Wochen Durchfall oder einen **fieberhaften Infekt** (Fieber über 38°C) oder Kontakt zu Personen mit **Infektionskrankheiten (z.B. Grippe, Masern, Mumps)**? | ☒ | ☒ |
| 13. | Haben Sie sich in den letzten 4 Monaten **tätowieren** oder **piercen** lassen oder tragen Sie ein **Permanent-Make up**? | ☒ | ☒ |
| 14. | Wurden Sie in den letzten 4 Monaten außerhalb einer medizinischen Einrichtung **akupunktiert** oder in das Ohr gestochen? Hatten Sie eine **Schnitt-** oder **Stichverletzung** mit „verunreinigten" medizinischen Instrumenten? | ☒ | ☒ |
| 15. | Hatten Sie in den letzten 4 Monaten unklares Fieber, Lymphknotenschwellung, Nachtschweiß oder unklaren Gewichtsverlust? | ☒ | ☒ |
| 16. | Waren Sie in den letzten 4 Monaten im Krankenhaus oder in einer medizinischen Einrichtung (z.B. schwerer Unfall, größere Operation, **Magen-** oder **Darmspiegelung**, Endoskopie, Therapie über zentralen Venenzugang, Verweilkatheter)? | ☒ | ☒ |
| 17. | Hatten Sie in den letzten 4 Monaten engen **infektionsgefährdeten Kontakt** zu Personen (z.B. Lebensgemeinschaft oder familiäre Pflege), die an **Hepatitis B, Hepatitis C, HIV oder Tuberkulose** leiden? | ☒ | ☒ |
| 18. | Hatten Sie eine **Herz-/Kreislauferkrankung** (Bluthochdruck, Herzschwäche, Herzinfarkt, Herzrhythmusstörungen, Schlaganfall, Durchblutungsstörungen, Thrombosen)? | ☒ | ☒ |

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Kostenlose Info-Hotline 0800 190 190 oder im Internet unter http://www.roteskreuz.at/ooe

A

Bitte wenden!

| | Bitte Zutreffendes ankreuzen! | Ja | Nein |
|---|---|---|---|
| 19. | Leiden oder litten Sie an einer **chronischen Magen/Darmerkrankung, Asthma, Autoimmunerkrankung, Blutungsneigungen, Diabetes, Epilepsie, Krebs, Nierenerkrankung, Rheuma, Osteomyelitis, neurologische Erkrankung?** | ☒ | ☒ |
| 20. | Haben Sie jemals **Blutkonserven** und/oder **Gerinnungsfaktoren** erhalten? | ☒ | ☒ |
| 21. | Waren Sie in den letzten 6 Monaten im **Ausland?** (Tagesausflüge oder Einkaufsfahrten in das benachbarte Grenzgebiet müssen hier nicht angeführt werden) Wenn ja, wo? | ☒ | ☒ |
| | Sind Sie in einem **Malariagebiet** geboren, bzw. aufgewachsen oder haben Sie sich dort länger aufgehalten? | ☒ | ☒ |
| | Waren Sie in den letzten 4 Wochen in einem **Westnil-Virus**-Infektionsgebiet wie zum Beispiel in einem aktuellen europäischen Risikogebiet oder USA, Mexiko, Kanada, Israel? | ☒ | ☒ |
| 22. | Hatten Sie eine der folgenden Infektionen: **HIV, Leberentzündung (Hepatitis), Syphilis, Chagas-Krankheit, Q-Fieber, Leishmaniose, Lepra, Babesiose, HTLV-1/2, Rickettsiose, Schlafkrankheit, Malaria** oder andere? | ☒ | ☒ |
| 23. | Waren Sie in den letzten 2 Jahren an **Tuberkulose, Toxoplasmose, Brucellose, Borreliose** oder in den letzten 6 Monaten an **Mononukleose (Pfeiffersches Drüsenfieber)** erkrankt? | ☒ | ☒ |
| 24. | Wurde bei Ihnen oder einem Familienangehörigen jemals die **Creutzfeldt-Jakob'sche Erkrankung** oder eine andere von Prionen verursachte Veränderung (vCJD) vermutet oder festgestellt? | ☒ | ☒ |
| 25. | Haben Sie sich zwischen 1980 und 1996 insgesamt mehr als **6 Monate im Vereinigten Königreich Großbritannien und Nordirland** aufgehalten? Wurden Sie nach 1980 in diesem Land operiert? Haben Sie nach 1980 in diesem Land Blutkonserven oder Gerinnungsfaktoren erhalten? | ☒ | ☒ |
| 26. | Wurden Sie mit **menschlichen Wachstumshormonen** oder einem anderen **Hypophysenextrakt** behandelt? | ☒ | ☒ |
| 27. | Haben Sie sich einer **stereotaktischen Operation (Gehirneingriff mittels Sonde)** unterzogen? | ☒ | ☒ |
| 28. | Sind Sie Empfänger von **Organ-, Gewebe-, Hornhaut- od. Gehirnhaut-Transplantaten?** | ☒ | ☒ |
| 29. | Hatten Sie **Sex im Austausch für Geld oder Drogen?** Haben Sie (sich) **Drogen gespritzt oder geschnupft?** Waren Sie jemals einem (auch einmaligen) Risiko einer sexuell übertragbaren Infektion (z.B. Hepatitis B, Hepatitis C, HIV) durch Intimpartner mit Risikoverhalten ausgesetzt? z.B.: Hatten Sie als Mann Sex mit einem anderen Mann? Hatten Sie Geschlechtsverkehr mit Personen aus Ländern mit einer erhöhten AIDS-Rate? Bitte beachten Sie die aufliegende Information über HIV und den freiwilligen Selbstausschluss. | ☒ | ☒ |

| | | |
|---|---|---|
| Ich erkläre mich bis auf Widerruf damit einverstanden, dass meine personenbezogenen Daten für Zwecke des Roten Kreuzes verarbeitet werden, insbesondere auch für Spenden- und Werbezwecke (schriftlich, telefonisch, SMS, oder per E-Mail). | ☒ | ☒ |
| Ich bin damit einverstanden, dass mir Blut und Blutbestandteile entnommen werden. Das Blut wird zur Herstellung von Blutprodukten verwendet und auf verschiedene Krankheiten und gesundheitsrelevante Blutwerte getestet. | ☒ | ☒ |
| Restmaterial der Blutspende kann für wissenschaftliche Zwecke und zur Entwicklung neuer Testmethoden verwendet werden. | ☒ | ☒ |
| Die Hinweise auf mögliche Infektionskrankheiten (z.B. AIDS), Blutspendekomplikationen und den „freiwilligen Selbstausschluss" (siehe Spenderinformation) habe ich erhalten, gelesen und verstanden. | ☒ | ☒ |
| Ich bin über Spenderrisiken im Rahmen der Blutspende aufgeklärt worden. Ich hatte Gelegenheit Fragen zu stellen und meine Fragen wurden zur Gänze beantwortet. | ☒ | ☒ |

**Spender-Erklärung:**

Ich versichere, dass bei mir keiner der genannten Risikofaktoren vorliegt. Es ist mir bewusst, dass Infektionskrankheiten (z.B. HIV, Hepatitis B, Hepatitis C) durch mein Blut übertragen werden können. Ich habe alle Fragen nach bestem Wissen und Gewissen beantwortet.
Ich erkläre mich ausdrücklich damit einverstanden, dass die im Rahmen meiner Spende erhobenen persönlichen Daten, die erhobenen Blutbefunde sowie der Blutspenderfragebogen unter Berücksichtigung des Datenschutzes sowohl bei den vom Österreichischen Roten Kreuz betriebenen Blutspendeeinrichtungen, als auch bei der jeweiligen Blutbank des Krankenhauses gespeichert und verwendet werden dürfen, wobei die Daten in der Blutbank einen Teil der Krankengeschichte im Sinne des Krankenanstaltengesetzes darstellen können. Ich nehme zur Kenntnis, dass ich frühestens 30 Minuten nach der Blutspende aktiv am Straßenverkehr teilnehmen darf.
Sollten sich bei mir Symptome einer Erkrankung zeigen, werde ich sofort die Blutspendeeinrichtung davon verständigen.

Herzlichen Dank für Ihre Bereitschaft zur Blutspende!

**Unterschrift Spender:**

**Spendetauglichkeit (vom Blutspendearzt auszufüllen)**

Spendefähigkeit im Sinne BSV 2008 Ja ☒ Nein ☒

**Paraphe des Blutspendearztes:** Version 11

**Anmerkungen zur ärztlichen Befragung, Eignungsuntersuchung, zu Komplikationen oder therapeutischen Maßnahmen**

(Dokumentation, zeitlicher Verlauf)

GFB V11

ÖSTERREICHISCHES ROTES KREUZ, LANDESVERBAND OBERÖSTERREICH; BLUTZENTRALE LINZ
BEH. KONZ. LABORATORIUM FÜR BLUTGRUPPENSEROLOGIE - BEH. KONZ. AMBULATORIUM FÜR BLUTTRANSFUSION
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B

# 9.3 Ethics

ethik kommission
Medizinische Universität Wien

Borschkegasse 8b/6
1090 Wien, Österreich
T +43(0)1 404 00-21470, 22440
F +43(0)1 404 00-16900
ethik-kom@meduniwien.ac.at
http://ethikkommission.meduniwien.ac.at/

**Votum:**

**EK Nr: 1235/2018**
**Projekttitel:** Bestimmung der Sekretions-Spiegel Antimikrobieller Peptide von Peripheren Mononukleären Zellen (PBMCs) nach Inkubation mit Methylenblau.
**Antragsteller/in:** Herr Martin Leopold Direder
**Institution:** Universitätsklinik für Chirurgie, Forschungslabor, FFG-Projekt APOSEC
**Sponsor:** Medizinische Universität Wien

Teilnehmende Prüfzentren:

| Ethik-Kommission | Prüfzentrum | Prüfärztin/arzt |
|---|---|---|
| Ethikkommission der Medizinischen Universität Wien | Medizinische Universität Wien, Abteilung für Chirurgie, Forschungslabor | Herr Univ. Prof. Priv. Doz. Dr. med Hendrik Jan Ankersmit |

Die Stellungnahme der Ethik-Kommission erfolgt aufgrund folgender eingereichter Unterlagen:

Conflict of Interest

| Name | Version | Datum |
|---|---|---|
| Conflict of Interest_1.2 | 1.2 | 28.02.2018 |

Lebenslauf (CV)

| Name | Version | Datum |
|---|---|---|
| WWTF_CV Ankersmit | 1 | 28.02.2018 |

Sonstige

| Name | Version | Datum |
|---|---|---|
| Screenshot Diplomarbeitseintrag | 1 | 28.02.2018 |
| Verpflichtungserklärung | 1 | 28.02.2018 |
| DA-Anmeldungsbestätigung ETHIK_Direder | 1 | 27.07.2018 |

Studienprotokoll (Prüfplan)

| Name | Version | Datum |
|---|---|---|
| Project plan_DM 1.2 | 1.2 | 28.02.2018 |

**Die Kommission fasst folgenden Beschluss (mit X markiert):**

| | |
|---|---|
| [X] | Es besteht kein Einwand gegen die Durchführung der Studie.<br><br>ACHTUNG: Unter Berücksichtigung der "ICH-Guideline for Good Clinical Practice" gilt dieser Beschluss ein Jahr ab Datum der Ausstellung. Gegebenenfalls hat der Antragsteller eine Verlängerung der Gültigkeit rechtzeitig zu beantragen. |

**Ergänzende Kommentare der Sitzung am 10.04.2018:**

Andere:
Das unterschriebene Antragsformular ist in Papierform nachzureichen.

**Ergänzende Kommentare:**

Nachtrag vom 2. August 2018:
Die Antragsteller legen am 17.04.2018 das unterzeichnete Antragsformular vor, das von der Ethik-Kommission akzeptiert wird. Die Anmeldebestätigung der Studienabteilung wird am 27.07.2018 hochgeladen.

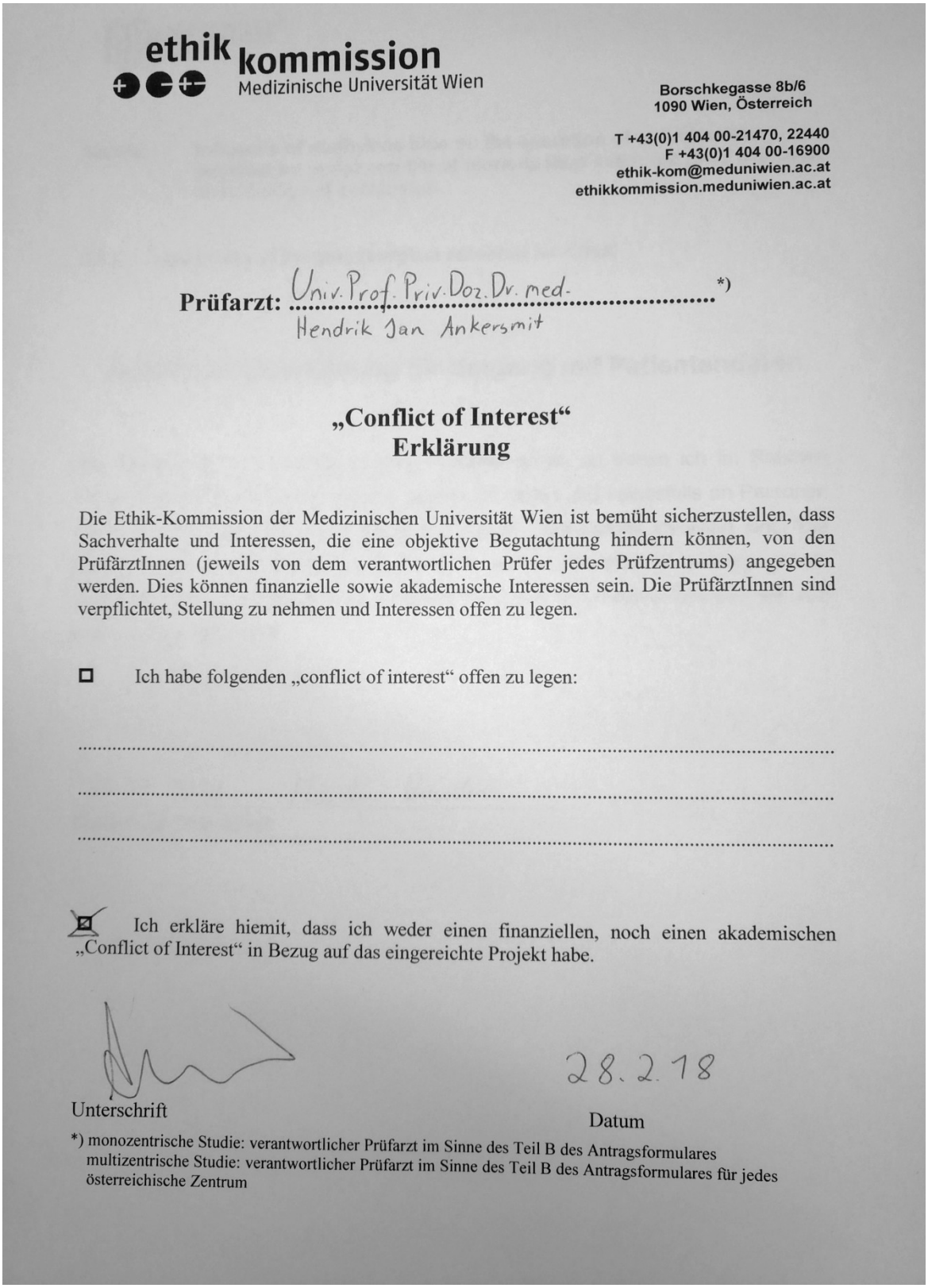
Die aktuelle Mitgliederliste der Ethik-Kommission ist unter folgender Adresse abrufbar:
http://ethikkommission.meduniwien.ac.at/ethik-kommission/mitglieder/

Mitglieder der Ethik-Kommission, die für diesen Tagesordnungspunkt als befangen anzusehen waren und daher laut Geschäftsordnung an der Entscheidungsfindung/Abstimmung nicht teilgenommen haben: **keine**

Dieses Dokument ist für berechtigte Benutzer/innen in digitaler Form unter folgender Adresse abrufbar:
https://ekmeduniwien.at/vote/14290/download/

| | | |
|---|---|---|
| SIGNATURINFORMATIONEN | **Unterzeichner** | Dr. Jürgen Zezula |
| | **Datum/Zeit-UTC** | 2018-08-02T15:26:17Z |
| | **Prüfinformation** | Informationen zur Prüfung der elektronischen Signatur finden Sie unter: https://www.signaturpruefung.gv.at |

# 9.4 Conflict of Interest



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**Prüfarzt:** Univ. Prof. Priv. Doz. Dr. med. Hendrik Jan Ankersmit *)

**„Conflict of Interest"**
**Erklärung**

Die Ethik-Kommission der Medizinischen Universität Wien ist bemüht sicherzustellen, dass Sachverhalte und Interessen, die eine objektive Begutachtung hindern können, von den PrüfärztInnen (jeweils von dem verantwortlichen Prüfer jedes Prüfzentrums) angegeben werden. Dies können finanzielle sowie akademische Interessen sein. Die PrüfärztInnen sind verpflichtet, Stellung zu nehmen und Interessen offen zu legen.

☐ Ich habe folgenden „conflict of interest" offen zu legen:

.....

.....

.....

☒ Ich erkläre hiemit, dass ich weder einen finanziellen, noch einen akademischen „Conflict of Interest" in Bezug auf das eingereichte Projekt habe.

Unterschrift

28.2.18
Datum

*) monozentrische Studie: verantwortlicher Prüfarzt im Sinne des Teil B des Antragsformulares
multizentrische Studie: verantwortlicher Prüfarzt im Sinne des Teil B des Antragsformulares für jedes österreichische Zentrum

# 10 Curriculum vitae

Martin Direder

| | |
|---|---|
| Born: | 15th October, 1993, Tulln, Austria |
| Parents: | Ing. Leopold Direder<br>Marianne Direder, maiden name: Preisinger |
| Siblings: | Daniela Direder, M.A.<br>Georg Direder |
| Citizen: | Austria |
| Home address: | Kirchengasse 7<br>3465 Unterstockstall<br>Austria |

**Email:** M.direder@gmx.at

| | |
|---|---|
| Education: | graduated 2013 at the Private Höhere Technische Lehranstalt für Lebensmitteltechnologie und Fleischwirtschaft Hollabrunn with distinction |
| | Social Service at the Red Cross Tulln, Lower Austria<br>10/2013 – 06/2014 |
| | Medical University of Vienna<br>Human medicine<br>10/2014 – present |
| | Tutor for anatomy course<br>Center of Anatomy and Cell Biology,<br>Medical University Vienna<br>SS 2017 |
| | Research Fellow at the Department of Surgery, General Hospital Vienna, Medical University of Vienna, Austria<br>05//2017 – present |
| Clinical Training: | Clinical Clerkship at the Department of Psychiatry and Psychotherapy<br>Klinikum rechts der Isar, München<br>08/2018 |
| | Clinical Clerkship at the Department of Trauma Surgery<br>Universitätsklinikum Krems<br>09/2017 |

Clinical Clerkship at the Department of Emergency Medicine
Universitätsklinikum Krems
09/2017

Clinical Clerkship at the Department of Children Surgery
SMZ Ost Donauspital
08/2017

Clinical Clerkship at the Department of Internal Medicine
Universitätsklinikum Tulln
07/2016

Clinical Clerkship at the Department of Orthopedics
Universitätsklinikum Krems
07/2015

Congresses and Meetings: Arbeitsgemeinschaft für Notfallmedizin; Notfallsymposium; Graz; 20.05.2017

31st annual meeting of the European Association for Cardio-Thoracic Surgery; Translational and Basic Science Course; Vienna; 7.-8.10.2017

PACT Joint Symposium "Regenerative Medicine: Taking the Science to the Patient"; Vienna; 30.-1.12.2017

Awards and Grants: Löffler-Müller-Stipendium für ausgezeichnete Leistungen 2008/09

Löffler-Müller-Stipendium für ausgezeichnete schulische Leistungen 2009/10

Löffler-Müller-Stipendium für ausgezeichnete schulische Leistungen 2010/11

Löffler-Müller-Stipendium für ausgezeichnete schulische Leistungen 2012/13

Language skills: Native German Speaker
Proficient in English

Computer Skills:

Volunteering: Paramedic at Austrian Red Cross since 2012
Member of the voluntary Fire Brigade since 2010


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