Diplomarbeit

Effects of Blood Sample Handling Procedures on Measured Cytokine and Chemokine Concentrations in Human Serum and Plasma

zur Erlangung des akademischen Grades

Doktor der gesamten Heilkunde
(Dr.med.univ.)

an der

Medizinischen Universität Wien

ausgeführt an der

Universitätsklinik für Chirurgie

unter der Anleitung von

Ass.-Prof. Univ.-Doz. Dr. Hendrik Jan Ankersmit

eingereicht von

Lucian Beer

Mat.Nr.: 0842107

Stranzendorf, am 25.3.2012 …….....

(Unterschrift)

Danksagung

Ich möchte mich bei all jenen bedanken, die mich bei der Verfassung dieser Arbeit durch ihren fachlichen Rat oder ihre persönlichen Worte unterstützt und motiviert haben.

Besonderer Dank gilt Ass.-Prof. Univ.-Doz. Dr. Hendrik Jan Ankersmit, der mir die Möglichkeit gab Einblicke in die Welt der Wissenschaft zu gewinnen und mich im Rahmen dieser Arbeit mit großer Sorgfalt betreut hat. Weiters möchte ich mich bei meinem LaborkollegenInnen bedanken, im Besonderen bei Dr. Michael Lichtenauer PhD und Michael Mildner die mich bei der Ausführung dieses Projekts unterstützt haben.

Ganz besonderer Dank gilt meinen Eltern Gabriele und Rudolf, die mir dieses Studium ermöglicht haben und sowohl in persönlicher als auch beruflicher Beziehung als Vorbilder meiner Entwicklung dienten. Auch meiner Schwester Patricia und meinen Großeltern danke ich für die ermunternden und erhellenden Worten in den unzähligen Gesprächen nach langen Forschungstagen.

Table of Contents

1.1 Abstract

Cytokines are small protein molecules that are secreted by different cell types. Over the last decades the analysis of cytokine concentrations in serum and plasma has become an important research issue in several disease conditions. However, cytokine concentrations are usually not considered to be very stable after blood collection, which might therefore alter test results. A known source of error is the time period between blood withdrawal, arrival in the laboratory and processing of the sample.

This study was intended to evaluate the influence of handling and storage conditions on detectable cytokines in human serum and plasma tubes. Furthermore, triggers for cytokine release from peripheral blood mononuclear cells (PBMC) were analyzed.

Venous blood was taken from healthy subject using different blood tubes (serum, heparin plasma, and EDTA plasma). Blood tubes were either centrifuged initially within 30 minutes after venipuncture and kept frozen at -80°C until further testing or were stored at 4°C, at room temperature and at 37°C for 4 and to 24 hours prior to centrifugation. Cytokine stimulatory potential of serum and plasma was evaluated by incubation of PBMC with increasing concentration of autologous serum, autologous plasma or fibrin. Samples were evaluated for different cytokines using commercially available enzyme-linked immunosorbent assay (ELISA) kits.

All examined mediators rose when samples were stored above room temperature for more than 4 hours in serum tubes. This rise of serum cytokine levels culminated in a 334-fold increase for IL-8, a 79-fold increase for IL-6, a 22-fold increase for ENA-78, and 17-fold increase for GCP-2 compared to basic values. A similar increase of cytokine concentrations could not be examined in plasma tubes. Cytokine concentrations rose in a dose dependent manner in supernatants of PBMC incubated with autologous serum.

These data indicate that most cytokine concentrations remain stable when analyzed within a short interval after venipuncture. When tubes were exposed to temperatures higher than room temperature, levels of all measured cytokines increased.

1.2 Zusammenfassung

Zytokine sind kleine Proteine welche von einer Vielzahl verschiedener Zellarten produziert werden. In den letzten Jahrzehnten gewann die Messung von Zytokinkonzentrationen in Serum- und Plasmaproben bei verschiedenen Krankheitsbildern zunehmend an Bedeutung. Zytokinkonzentrationen gelten jedoch als äußerst variabel in Blutproben, wodurch die Messergebnisse verfälscht werden. Eine bekannte Fehlerquelle ist die Zeitspanne zwischen Blutabnehme, dem Eintreffen im Laboratorium und der Weiterverarbeitung der Blutprobe.

Ziel dieser Studie war es den Einfluss von Lagerungsdauer und Lagerungszeit von Blutproben, auf die in diesen Blutproben gemessenen Zytokinkonzentrationen zu untersuchen. Weiters wurde die Zytokinfreisetzung von peripheral blood mononuclear cells (PBMC) durch bestimmte Triggerfakoren untersucht.

StudienteilnehmerInnen wurde venöses Blut in Serum- und Plasmaröhrchen (Heparin, EDTA) abgenommen. Diese Blutröhrchen wurden innerhalb 30 Minuten nach der Blutabnahme zentrifugiert und Serum/Plasma bei -80°C bis zu weiteren Tests tiefgefroren. Die verbleibenden Blutröhrchen wurden bei 4°C, Raumtemperatur und 37°C für 4 bzw. 24 Stunden gelagert, anschließend zentrifugiert und Serum/ Plasma abgehoben. Um den stimulatorischen Effekt von Serum- und Plasmaproben zu untersuchen wurden PBMCs mit ansteigenden Dosen von autologem Serum, Plasma oder Fibrin inkubiert. Die Proben wurden mittels Enyme-linked Immunosorbent Assay (ELISA) Kits ausgewertet.

Es zeigte sich ein Anstieg aller untersuchter Mediatoren bei Lagerung der Proben bei 37°C für mehr als 4 Stunden im Serumröhrchen. Es kam zu einem 334-fachen Anstieg von IL-8, einem 79-fachen Anstieg von IL-6, einem 22-fachen Anstieg von ENA-78, einem 17-fachen Anstieg von GCP-2 verglichen mit den Ausgangswerten. In Plasmaröhrchen konnte kein ähnlicher Anstieg der Zytokinkonzentrationen gemessen werden. Zytokinkonzentrationen im Zellüberstand von PBMCs, welche mit ansteigenden Dosen autologen Serums inkubiert wurden, stiegen dosisabhängig an.

Diese Daten zeigen, dass Zytokinkonzentration stabil erscheinen, wenn die Proben innerhalb kurzer Zeit nach Blutabnahme verarbeitet werden. Wenn Proben jedoch

über Raumtemperatur gelagert werden, kommt es zu einem Anstieg aller gemessener Zytokine.

2 Introduction

2.1 Cytokine

The term cytokine was introduced by Stanley Cohen in 1974. Until then the term lymphokine, had been used to describe protein mediators mainly produced by lymphocytes[1]. By definition, cytokines are peptide mediators which modulate aspects of immunity and inflammation. The cytokine family consists of a large number of secreted factors involved in intercellular communication, which regulate essential function such as wound healing, host defense, homeostasis and hematopoiesis. They exert multiple biological activities depending on cell type, timing and context [2].

Cytokines share similarities with hormones since both regulate homeostasis and are synthesized in one organ/cell. A difference between cytokines and hormones is that hormones have higher circulation levels compared to cytokines. Circulating levels of many cytokines are below the detection limit of the assay[2].

2.2 Cytokine metabolism

A wide range of different stimuli are described that induce cytokine release *in vivo* and *in vitro* using both animal and human models. The cytokine network consists of complex inter-relationships between different type of cytokines, indicating that a simple cascade model where one cytokine can induce the secretion of another cytokine does not describe the mechanism accurately[2].

The *in vivo* half-life of cytokines injected intravenously is usually stated in minutes, whereas the half-life of cytokines in tissue culture condition is generally greater due to the lack of hepatic or renal clearance[3].

A general characteristic of cytokines is their molecular weight of less than 30 kilodaltons.

Due to special structures and function cytokines can be clustered into several classes[1]:

- interleukins
- tumor necrosis factors

- interferons
- colony stimulation factors
- transforming growth factors
- chemokines

2.3 Cytokines in human disease

Methods to evaluate cytokine concentration in different sample types improved during the last decades. Due to user-optimized assays the role of cytokines was investigated in a wide range of different diseases. Cytokine alterations are observed after acute injury[4-6], sepsis[5, 7, 8], chronic diseases[1, 9], autoimmune disease[10-12] and cancer[12].

2.4 Chemokines

The chemokine system plays a pivotal role in the regulation leukocyte movement to sites of infection and tissue damage. During the last decades it had been shown that chemokines are involved in gene transcription, homeostasis, angiogenesis, fibroproliverative disorders, wound repair, atherosclerosis and malignancy[13-15].

In 1977 platelet factor 4 (PF-4, CXCL4) was the first classified chemokine. The second chemokine, Interferon gamma-induced protein 10 (IP-10, CXCL10), was identified in 1985, following by several other chemokines isolated from tissues or cell culture supernatants[16].

2.4.1 Structure

More than 47 chemokines have now been identified in humans. Chemokines are a superfamily of homologous 8- to 10-kDa heparin-binding cytokines molecules. They are small proteins, usually 70-120 residues long that contain 1-3 disulfides. The structure consists of a short N-terminal region, an extended N-loop region, followed by three β-sheets and an α- helix (Fig. 1)[17].

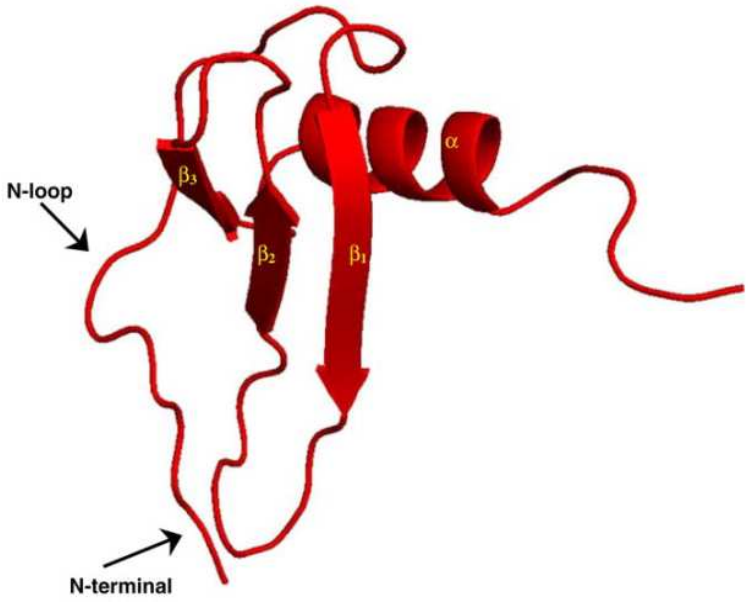

Figure 2.1 3D or tertiary structure imagine of chemokine structure with one alpha-helix and 3 antiparallel beta-sheets[18].

Chemokines feature very similar secondary and tertiary structures with 20-40% homology across the whole superfamily, whereas the sequence homology is highly variable.

Depending on their different structure (on the basis of the presence of variation on a conserved cysteine motif in the mature sequence of proteins) chemokines can be divided in four subfamilies.

The CC chemokine family (so called because of the juxtaposition of two cysteine residues) is composed of 28 members.

Members of the CXC family are characterized by a pair of cysteine, which are separated by one amino acid, represented by the letter X. It includes 16 ligands and 8 receptors in humans. It is suspected that this redundancy may allow chemokine / receptor pairs to play an exceptional fine-tuning role for the immune system[19]. The CXC chemokine structure consists of a disordered N-terminus of 6-10 amino acids followed by a long N-loop, with a three-stranded anti parallel β-sheet that ends in a 3-10 C-terminal helix. The N-loop possesses important binding function, while the N-terminus functions as a key signaling domain[17]. This family can be further subdivided on the basis of presence or absence of another three aminoacid sequence of the so called 'ELR' motif (the amino acid sequence glutmic acid-leucine-argenine), located directly proximal to the CXC sequence. It has been shown that the ELR motif is

essential for chemotactic and angiogenic activity[20]. Depending on the presence or absence of the ELR motif in their amino terminal end, CXC chemokines are potent inhibitors or promoters of angiogenesis [21].

The third group of chemokines is called the C chemokine group with only 1 member named lymphotactin (XCL1). It has two cysteines (one N-terminal and one cysteine downstream).

The CX3C family consists only of one chemokine called fractalkine (CX3CL1). Fractalkine is a large protein of 373 amino acids containing multiple domains which are structurally distinct from other chemokines. Compared to other chemokines fractalkine has a long mucine-like stalk (residues 77 to 317) with predicted O-glycosylated serine and threonine and transmembrane domain (residues 337 to 373). The membrane-bound form functions as an adhesion molecule and promotes shear-resistant adhesion of CX3C-Receptor 1 leukocytes, while the soluble form consist of the chemokine domain and the extracellular mucine like stalk[22, 23].

2.4.2 Chemokine receptors

Chemokine receptors are known to be G-protein-coupled receptors (GPCRs)[24]. The first chemokine receptors were cloned in 1991. They consisted of two heptahelical proteins with a high attraction to interleukin 8 (IL-8, CXCL8)[25]. To date seven receptors for the CXC chemokines and ten receptors for the CC chemokines are described. Furthermore, there is a single receptor for fractalkine and the two receptors the C chemokines[26].

As mentioned before most chemokine receptors are able to bind more than one chemokine. However, a distinct specificity exists, as CC receptors can only bind chemokines with a CC motive and CXC receptors can only tag to CXC chemokines. This so called “ligand-receptor restriction” may be related to structural differences between CC and CXC chemokines, which have similar secondary and tertiary structures (see Figure 2.1) but different quaternary structures[15, 18].

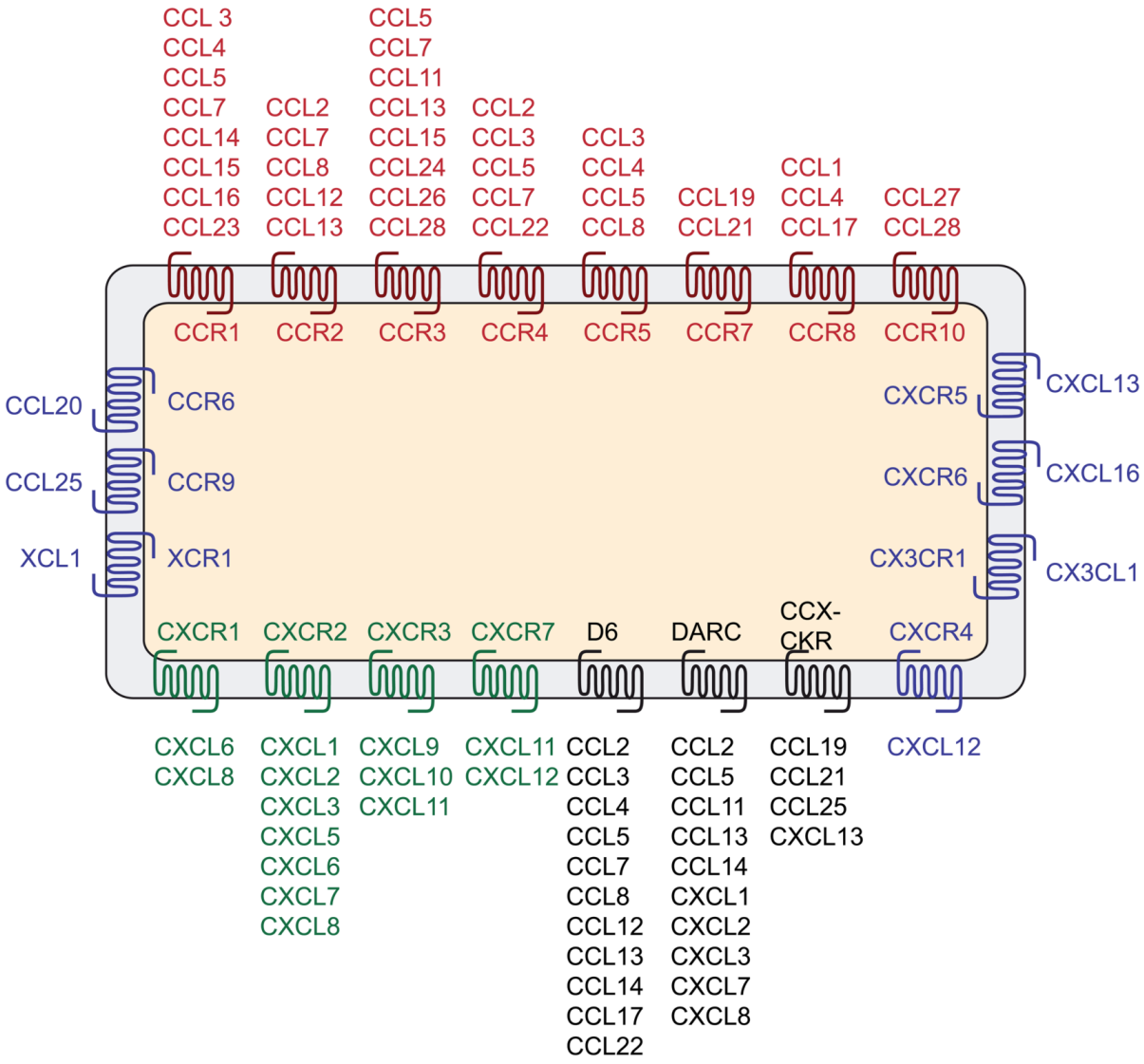

Figure 2.2 Chemokine receptors and their ligands[26].

2.4.3 Chemokine nomenclature

In the last century chemokines were named randomly with no clear system being used. The same proteins were often reported under different names. Some groups named chemokines after the function of the protein, while others were named according to the cell type that secretes the chemokine, e.g. platelet factor 4. This caused confusion, even among scientists working in the field.

In 2000 a systematic nomenclature of chemokines and chemokine receptors was published to solve the confused nomenclature associated with chemokines. The nomenclature is outlined in the following table. Beside the new systemic names, the historical nomenclature and the receptors for each chemokine are given in the table.

Systematic Name	Human Ligand	Chemokine Receptor(s)
CXC Chemokine/Receptor Family		
CXCL1	GRO-α	CXCR2
CXCL2	GRO-β	CXCR2
CXCL3	GRO-γ	CXCR2
CXCL4	PF4	Unknown
CXCL5	ENA-78	CXCR2
CXCL6	GCP-2	CXCR1, CXCR2
CXCL7	NAP-2	CXCR2
CXCL8	IL-8	CXCR1, CXCR2
CXCL9	Mig	CXCR3
CXCL10	IP-10	CXCR3
CXCL11	I-TAC	CXCR3
CXCL12	SDF-1α/β	CXCR4
CXCL13	BLC/BAC-1	CXCR5
CXCL14	BRAK/bolekine	Unknown
C Chemokine/Receptor Family		
XCL1	Lymphotactin	XCR1
XCL2	SCM-1β	XCR1
CX_3C Chemokine/Receptor Family		
CX3CL1	Fractalkine	CX3CR1
CC Chemokine/Receptor Family		
CCL2	MCP-1	CCR2
CCL3	MIP-1α	CCR1, CCR5
CCL4	MIP-1β	CCR5
CCL5	RANTES	CCR1, CCR3, CCR5
CCL7	MCP-3	CCR1, CCR2, CCR5
CCL8	MCP-2	CCR1, CCR2, CCR3, CCR5

Table 2.1 Chemokine nomenclature: systematic name, historical name and chemokine receptors; adapted from[22]

2.4.4 Withdrawal of blood

During the last century improvements were achieved in the development of new blood withdrawal systems. In the 19th century most syringes had been made of metal, glass or hard rubber. A disadvantage of these syringes was that the span of life of such products was very short, due to the weak resistance of the material during sterilization process.

At the beginning of the 20th century the first hypodermic syringes made of glass were produced by the Becton Dickinson Company. A big step forward was made in 1920 by the design of the first interchangeable syringes. However, there was still the problem to sterilize the syringes.

It took another 30 years to solve this problem. After Wald War II the Becton Dickinson Company developed replaceable, property fitting syringes.

At the same time Joseph J. Kleiner developed the Vacutainer blood collection tube. The innovation of this product was that the needle could be left in the vein while vacuum tubes were exchanged. In brief, the system consisted of a double-pointed needle, an evacuated tube and a holder for fitting the two together. While the long needle causes the venipuncture the short needle is pressed against the stopper. The blood pressure and the vacuum in the tube aspirate the blood once the tube is transfixed[27].

Beside the Vacutainer system, the syringe-needle technique is generally used. This technique offers the opportunity to control the intake of blood into the tube manually.

Additive	Inversion at Blood Collection	Laboratory Use
clot activator and gel for serum separation	5	clinical chemistry testing of serum for infectious disease blood clotting time: 30 minutes
lithium heparin and gel for plasma separation	8	clinical chemistry
silicone coated (glass) clot activator, silicone coated (plastic)	0 5	clinical chemistry blood clotting time: 60 minutes
thrombin-based clot activator	8	for stat serum determination in chemistry blood clotting time: 5 minutes
sodium heparin lithium heparin	8 8	clinical chemistry
liquid K3 EDTA (glass) spray-coated K2 EDTA (plastic)	8 8	K2 EDTA and K3 EDTA for whole blood hematology determination. K2 EDTA may be used for routine immunohematology testing, and blood donor screening
buffered sodium citrate 0.109 M (3,2%) plastic	3-4	for coagulation determinations. CATD for selected platelet function assays and coagulation determination, requires full draw

Table 2.2 Blood collecting tubes in clinics. Adapted from[28, 29]

Independent of the method used, there are rules to follow to generate valid results. The Clinical And Laboratory Standards Institute is an organization with the aim to generate good manufacture principles in laboratory work. In 2006, the sixth edition of a blood collecting and blood sample handling manual was published describing the procedure of a venipuncture as well as venipuncture supplies and sources of error during the venipuncture[30].

Firstly, tubes containing anticoagulant must be filled to the marked level to achieve the right proportion. Otherwise, the anticoagulation will be high and falsify the results.

Secondly, blood tubes should be collected in the following sequence: blood culture bottle – non-additive tube – sodium citrate – heparin – EDTA and at last oxalate fluoride. It is important to follow this order and to avoid cross contamination between additive tubes[30].

2.4.5 Preparation of serum

Blood serum develops after clotting of blood. It neither contains any cellular nor clotting factor components. To obtain serum, blood must be collected in a plain tube. After the coagulation process, which takes up to one hour, blood must be centrifuged to separate cellular components and serum.

To accelerate the clotting process to 10-15 minutes, many tubes contain clot-activating-devices. The most established clot-activating-devices are glass or silica particles. However, thromboplastin has also been used to achieve fast clotting. At benefit of these consumables is a lesser contamination of late forming fibrin. Furthermore, haemolysis is less likely when clotting activators are used.

2.4.6 Serum separators

To achieve a more accurate and faster separation of serum, special silicon gels or polyester formulations can be used. These gels exhibit a special density that is intermediate between the density of cells and the one of serum. The semi fluid gel changes its stiffness during centrifugation. The cell-clot accumulates at the bottom while the serum stays at the top of the tube. The best temperature for centrifugation is at 20-25°C because cooling may impair the flow characteristics while too high temperatures may cause a breakdown of the gel.

2.4.7 Preparation of plasma

Plasma contains clotting factors, but no cellular components.

To obtain plasma, anticoagulants have to be added to tubes that inhibit the clotting process. The clotting process can either reversibly or irreversibly be stopped. Commonly used anticoagulants in tubes are EDTA and heparin salts and sodium citrate. To achieve a consistent concentration of the anticoagulant, immediate mild mixing of the anticoagulant with the blood after venipuncture is necessary.

Depending on the platelet count three different types of plasma can be produced[31]

Plasma	Relative centrifugal force (g)	Centrifugation time (minutes)
Platelet rich	150-200	5
Platelet poor	1000-2000	10
Platelet free	2000-3000	15-30

Table 2.3 Platelet count in Plasma depends on centrifugal force and centrifugation time[31].

Too high temperatures or centrifugation for a long time will lead to haemolysis and falsification of plasma components.

Anticoagulants can interfere with some antibody-antigen reactions. Especially heparin reduces the reaction rate of some antibodies. Furthermore, it should not be used to determine cryoprotein levels. EDTA is known to inhibit the enzyme activity by chelating of calcium ions[32].

2.5 Cytokine and chemokine measurement

Over the last decades many different methods in analyzing cytokine concentrations or cytokine function in biological fluids have arisen due to the growing importance of these mediators in scientific life. The choice which test fits best depends on the expected cytokine concentration in the sample, the type of sample (fluid vs. tissue), the amount of the sample and financial aspects. In the following paragraphs frequently used techniques are described, without raising claim to completeness in the wide field of cytokine measurement[33].

2.5.1 Enzyme linked immunosorbent assays

Measurement of soluble cytokine or cytokine receptors in body fluids and cell supernatants are commonly based on enzyme linked immunosorbent assays (ELISA) or radioimmuno assays (RIA). ELISA was established in the 1970s and displays the most common method of cytokine measurement [34]. Usually double antibody sandwich ELISAs are used. A detection antibody linked to the bottom binds to its typical antigen, while another antibody linked to an enzyme provides detection and enhancement of the signal.

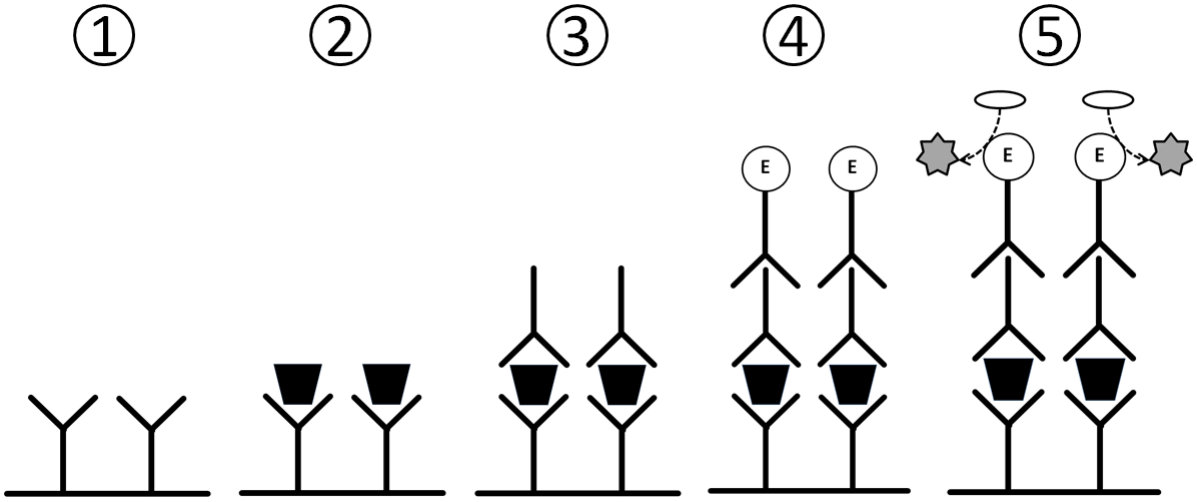

Figure 2.3 1: wells coated with capture antibody; 2: incubation with antigen (cytokine); 3: incubation with detection antibody; 4: incubation with enzyme (E); 5: addition of substrate and color reaction; ®Lucian Beer

Many manufacturers provide ELISA kits for a wide range of different cytokines. The sensitivity of an ELISA kit ranges between pictogram per milliliter to nanogram per milliliter over a 2 log working range[32, 35, 36].

However, a limitation of ELISA-based assays is that the read out is strongly dependent on antibody quality, kit manufacturer and experience of the operator. Furthermore, samples with cytokine concentrations above the dynamic range have to be diluted for the assay. This is eminently important when specimen above the dynamic range (need to be diluted) and samples within the dynamic range (do not require dilution) were measured in the same assay. It is well known that dilution reduces the concentration of the target cytokine as well as levels of cytokine inhibitors or binding proteins. Therefore, different dilution of samples may cause a measurement bias[32].

A few steps of the ELISA procedure can be semi-automated with robotics to reduce manual pipetting steps and avoid sources of random error. Assay automation is only cost-effective in larger studies or in clinical laboratories[36] and not commonly used in a clinical research setting.

Another disadvantage is that kits from different manufactures provide various results. Direct comparison between cytokine levels measured by different ELISA kits is therefore limited[37].

2.5.2 Multiplex arrays

Multiplex assays were produced for faster and more comprehensive cytokine profiling. The ratio of these assays is the purpose of measuring different cytokines in the same sample at one time point. The most established techniques are flow cytometric multiplex arrays, also known as bead-based multiplex assays (Figure 2.4).

2.5.2.1 Bead based assays

Multiplex assays contain a mixture of different bead labeled capture antibodies. After incubation samples with the capture-antibody beads, a further incubation step with florescence labeled detection antibodies is required to allow the detection of cytokines in fluorescence light. The amount of fluorescence is proportional to the concentration of the linked cytokine[32, 38].

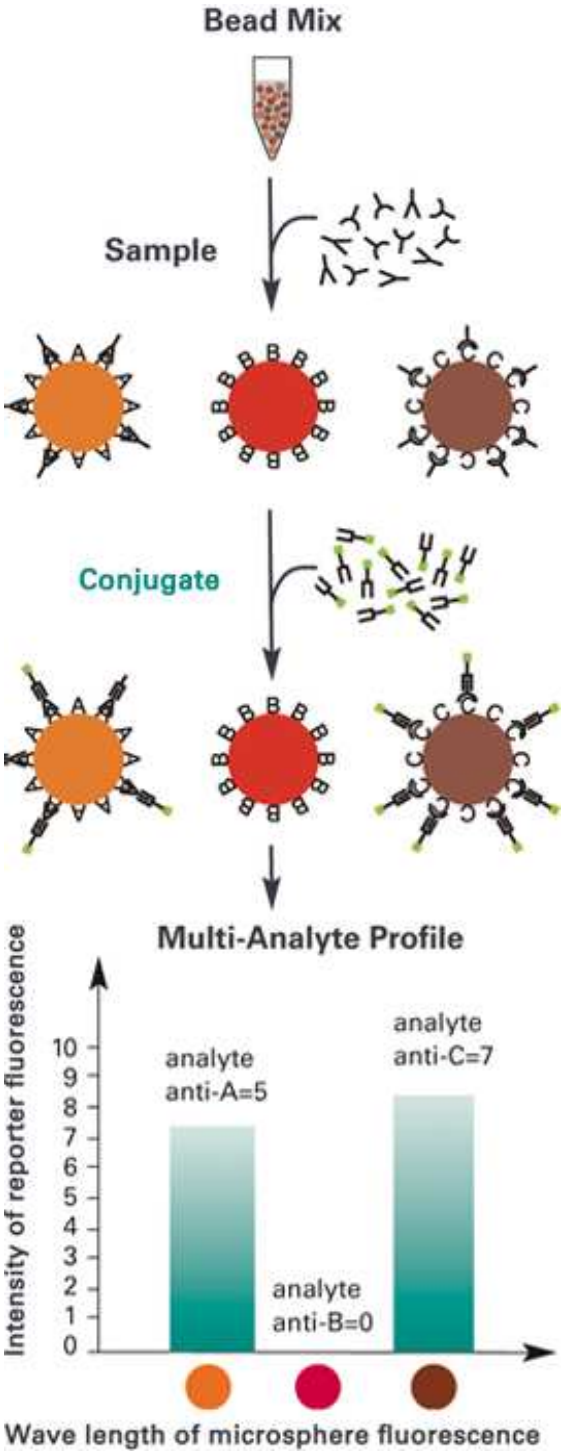

Figure 2.4 Principle of multiplex bead based arrays[39]

Up to 100 different proteins can be measured by this technique simultaneously. Based on this capacity the amount of sample (25-50 microliter) is clearly lower compared to ELISA techniques, where up to 100 microliters for one cytokine measurement are needed.

A further benefit of multiplex assays is the ability to detect different proteins across a wide dynamic range of concentration without the necessity to dilute samples[32, 33, 38].

However, there are some drawbacks that have to be mentioned:

- side-by side comparisons between ELISA results and multiplex assays are rare and therefore direct comparison between two different results might be difficult[40]
- cross reaction between different capture and detection antibodies as well as antigens in the test can influence the results
- type of sample (serum vs. plasma) influences results at multiplex assays whereas ELISA assays provide rather comparable results[32, 38].

2.5.2.2 Planar multiplex assays

An alternative to bead-based assays, planar based assays can be used to quantify protein concentration in samples. Multiplex planar array technologies are used in tumor research[41], immunology research[42, 43], and in scanning different body fluids for cytokine content[44].

Capture antibodies are immobilized on the solid support. Typically, sandwich assays are used. Samples containing proteins are incubated onto the array. Unbounded proteins are washed away. A labeled second antibody, not necessarily highly specific, is added for detection. After a further washing step the signal can be detected[45-47] (see figure 2.5).

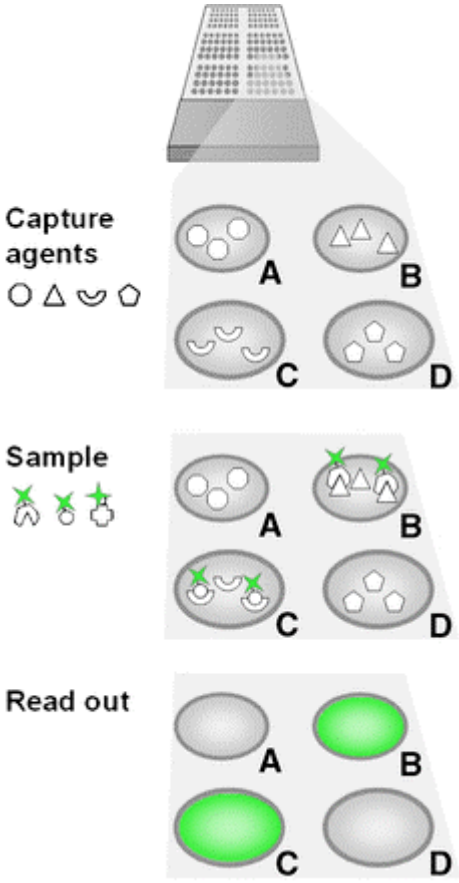

Figure 2.5 Principle of planar based multiplex arrays[48]: The sample is added to plated precoated with immobilized capture antibodies. Proteins bind to their cognate capture agent and the amount of protein can detected by a fluorescence read out.

Planar multiplex assays can simultaneously detect up to 50 different proteins. Samples do not need to be diluted prior to the test due to the great detection range of these assays. Therefore, multiples assays are suitable to screen different types of samples for cytokines and their characteristics.

A disadvantage of cytokine assays is that they do not provide quantitative results of protein amount as does an ELISA. However, it has been shown that ELISA and multiplex assays results correlate, indicating that multiplex assays provide valid results[44].

A further problem is to immobilize antibodies on the solid phase[45]. To use multiplex assays in diagnosis the manufactures have to prove that the antibody is immobilized. Due to expensive regulatory procedures, multiplex assays are not ruinously used in clinics[45].

2.5.3 Bioassays

Bioassays are a method to detect functional proteins and cytokine activity by adding cytokine samples to cells, tissue or animals[33]. These living materials act as an indicator system for the cytokine presence in the reviewed sample. Due to the type of assay the advantages are the high sensitivity (picogram per milliliter or less) and the ability to discover the effect of a cytokine on viable cells (e.g. stimulation of migration; induction of inflammatory function (interleukin-6) and inhibition of function (interleukin-1))[49].

Drawbacks of bioassays are the low specify, the requirement of cell cultures and the interference with unknown factors[50].

2.5.4 Single cell assays

Intra-cytoplasmatic cytokine staining and enzyme-linked immunospot (ELISpot) have been used to assess the *in vitro* cytokine production by different cells types. The technique of ELISpot assays is similar to ELISA assays. Cells are seeded on wells pre-coated with monoclonal antibodies specific for the cytokine of interest. During an incubation time the cells produce cytokines which are linked to the immobilized antibodies. After a washing step, an enzyme labeled antibody specific for the cytokine is added to the wells. By adding a substrate solution to the wells the color reaction appears and the area of cytokine expression can be measured[33, 51].

ELISpot assays are commonly used in the diagnosis of tuberculosis and in vaccine studies to monitor cellular responses[51, 52].

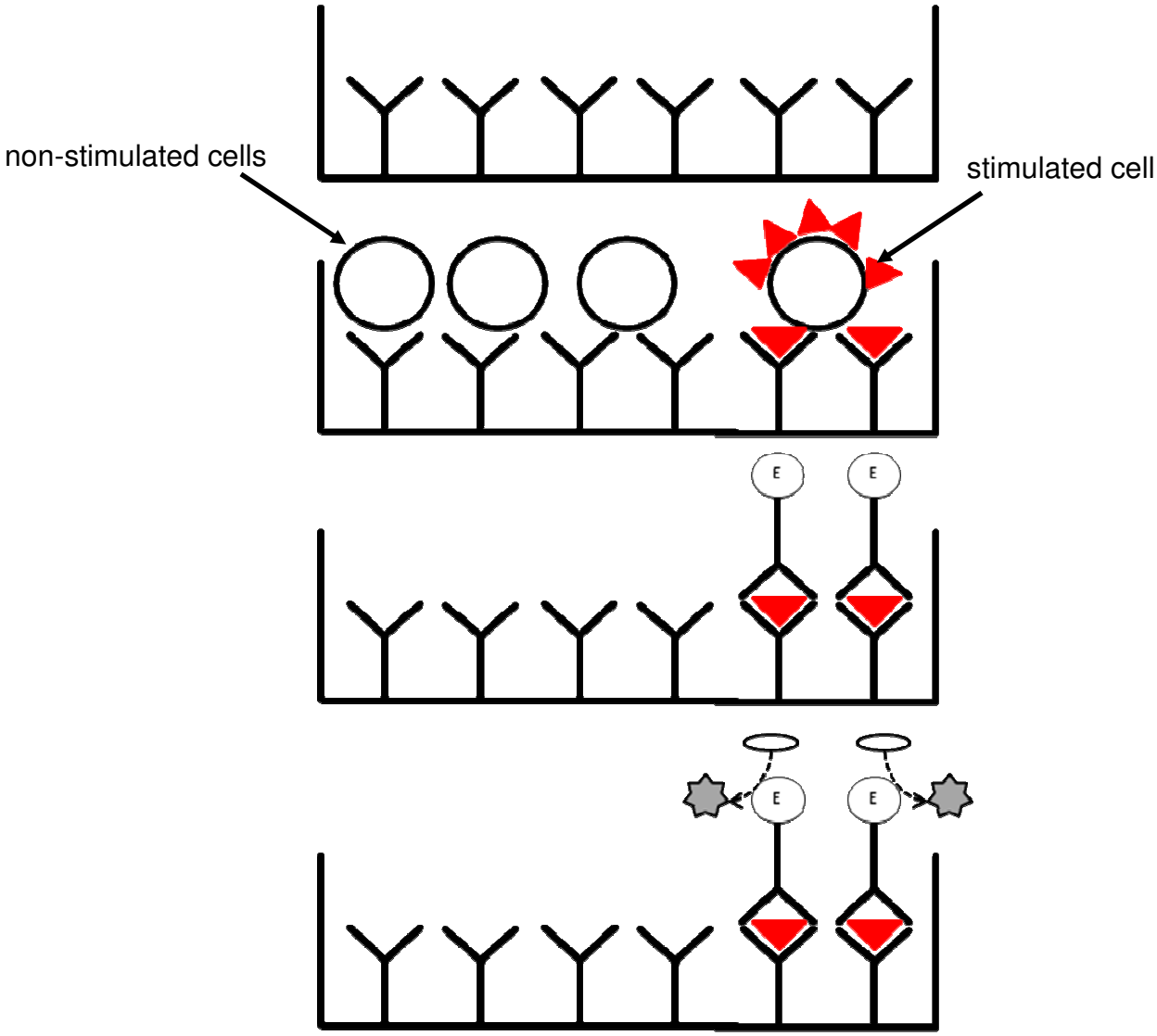

Figure 2.6 ELISpot**:** wells coated with capture antibody; stimulated cells secrete cytokines; incubation with detection antibody with enzyme (E); addition of substrate and color reaction; ®Lucian Beer

Beside the named methods, there are other methods for detection of cytokines in tissues (immunostaining and mRNA based assays) as well as DNA and protein microarrays, which are a promising technique of cytokine measurement in the future. Nevertheless, new technologies need to be used cautiously and further data are needed to be able to compare results generated by different methods among each other[32].

2.6 Measurement of cytokines and chemokines in clinical and basic research

Over the last decades the analysis of cytokine and chemokine levels in serum or plasma of patients has become an important research issue in several disease

conditions. A “PubMed” search done in November 2011 with the term “cytokine” and date of publication “2010” listed 35298 publications indicating the importance of cytokines in clinical research.

With the increasing measurement of cytokines in clinical studies an increasing variation of cytokine concentrations appeared in different publications. This phenomenon can be discussed exemplified by TNF-α concentrations examined in healthy subjects. TNF-α concentrations in healthy subjects range from low pg/mL to multiple ng/mL[53]. Therefore, attempts have been done to determine the average concentrations of cytokines in population based studies.

Multiplex assays enabled researchers to measure a high number of cytokines simultaneously in volumes of 25 to 50 µL. In 2011 a study assessed the concentration of three cytokines and C-reactive protein in 2884 healthy men and 3201 healthy women. The aim of this study was to establish a reference value and to determine influencing factors of cytokine concentrations in healthy subjects[54].

2.7 Factors of influence

2.7.1 Age / sex / BMI

Several studies indicate that the pro-inflammatory cytokines IL-6[55, 56], TNF-α and IL-1β[56] are impaired in immune cells taken from elderly people[57]. Men have greater IL-6 concentrations than women[58]. Postmenopausal women have significantly greater IL-6 levels compared with premenopausal women[59, 60]

These finding differs from those that have not shown any correlation between age and IL-6 concentrations[54, 61]

Higher BMI is associated with increased inflammatory cytokines[62, 63], whereas physical activity can have both, a positive or negative effect on IL-6 concentration [54].

2.7.2 Circadian rhythms

IFN-gamma, TNF-α, IL-1 and IL-12 production of stimulated whole blood cultures exhibit distinct circadian rhythms that peak in the morning and are inversely related to plasma cortisol concentration[64]. In patients with metastatic colorectal cancer a

significant daily rhythm of TNF-α concentration was found with a peak at 2 am and the nadir at 2 pm[64].

2.7.3 Correlation of cytokine levels in serum and plasma

Several reports in the literature examine the influence of serum and plasma samples of measurable cytokine concentrations[65, 66]. Furthermore, the anticoagulant used for collection of blood can affect the cytokine concentrations[65-69].

The platelet associated chemokines CCL3 (Macrophage inflammatory protein-1α – MIP-1α), CCL5 (RANTES), CXCL-8 (IL-8) CXCL4 (PF-4) and the cytokine transforming-growth factor β (TGF-β) can be elevated in serum levels by *ex vivo* degranulation of platelets[70, 71]. Serum samples contain higher concentrations of IL-1β, which is secreted from white blood cells during the clotting process[72]. Wong et al. examined that IL-8 concentrations are greater in serum samples than in acid citrate dextrose samples[67]. Levels of IL-2, IL-4, IL-5, IL-7, IL-10, IL-12p70, IL-13, IFN-gamma, G-CSF, HEP were lower in serum compared to plasma values[67]. These findings differ from those reported regarding that overall levels of cytokines were quite similar between different blood sample types[66].

2.7.4 Sample storage and stability considerations

The short half-life of cytokines, the secretion of cytokines by cells during blood storage and preparation, as well as the potential degradation of cytokines make the stability, treatment and storage of samples important factors in cytokine measurement and interpretation[73].

The period between blood withdrawal and centrifugation and between centrifugation and freezing as well as the time the samples are stored in a freezer can alter cytokine concentration.

A delayed processing of plasma levels is associated with the increase of IL-8, interleukin 1 Receptor antibody (IL-1Ra), matrix-metallopeptidase-9 (MMP9), whereas IL-12, CXCL10 (interferon gamma-induced protein 10 – IP-10), CCL2 (monocyte chemotactic protein-1 – MCP-1) and MIP-1α is reduced dependent on time [74]. Storage of plasma tubes at 37°C for 2 hours results in a significant increase of TNF-α [75]. Storage of plasma at 24°C for 20 days results in up to 55% lower TNF-α

concentrations compared to samples kept at 4℃ or -70℃ [76] indicating cytokine degradation .

The recommended long term storage temperature of samples for cytokine measurement is -80℃[77]. A current study showed that most cytokines stored at -80℃ are stable up to two years[77]. Different patterns were seen for different groups of cytokines. IL-13, IL-15, IL-17 ad IL-8 are significantly reduced after one year storage at -80℃, whereas IL-2, IL-4, IL-12 and IL-18 are stable for up to 4 years[77].

During a five-year storage, a five-fold increase as well as a decrease of different cytokine was found[78] indicating that, depending on the cytokine measurement cross reactivity between different protein epitopes can occur. These results indicate that storage stability differs in the measured cytokines and every cytokine needs to be considered on its own merits[76, 77].

Repeated freeze-thaw cycles can influence cytokine concentration. In a study published in the year 2000, TNF-α levels increased significantly after 3 freeze-thaw cycles[79], whereas other studies did not detect this effect[80]. Different results of these studies might be explained by the fact that the pathology of the patient from whom the sample has been obtained affects the result[53].

2.7.5 Sample preparation in clinical settings

Based on the multiple influence factors of cytokine concentrations a standard procedure should be adhered when blood samples are collected. These standards should include detailed patient instruction on preparing for the blood withdrawal, including diet and exercise restriction. Time of blood withdrawal as well as sample handling should be standardized to improve laboratory dependent procedures[32].

In the following paragraph general guidelines for handling blood samples are listed. Adapted from[32, 81]:

- The procedure is governed by the stability of the constituents of the sample. The most important causes for alterations to the quality of specimen are:
    - Metabolism of blood cells
    - chemical reactions

    - effect of light

- Short storage time improves the reliability of test results
- Cool storage conditions preserve reliability of laboratory results
- Cooled tubes should be used for blood collection
- Closed tubes should be used to store samples
- Separating agents improve the borderline between serum / plasma and cellular components
- Anticoagulants should be appropriately selected based on the desired assay and standardized
- Cross contamination between tubes containing different anticoagulants should be avoided
- Whole blood samples should be separated into serum or plasma after blood withdrawal
- Avoid repeated freeze-thaw cycles
- Thawing of samples should take place at room temperature
- Physical activity should be avoided prior to blood sampling
- New pipette tips should be used for aliquoting each sample to prevent contamination

3 Methods

3.1 Whole blood incubation experiment

This study was conducted in accordance with the local ethics committee and according to the principles of the Helsinki Declaration and Good Clinical Practice (EK: 2010/034). Informed consent was obtained from all participants in this study. Major inclusion criteria were body mass index 18-28 kg/m², no intake of anti-inflammatory drugs during the last two weeks, no acute infection during the last month, no chronic inflammatory disease and no physical activity over the last hours prior to participation in this study.

Venous blood was obtained from seven young healthy volunteers by venipuncture using blood collection tubes for serum, heparinized (lithium) plasma and EDTA plasma used in clinical setting (Greiner BioOne, Austria, sterile 9mL). To determine basic serum and plasma levels, selected tubes were centrifuged at 2330g for 15 minutes within 30 minutes after blood withdrawal. The obtained serum and plasma samples were stored at -80°. The remaining tubes were either kept in a refrigerator at 4°, at room temperature (24°) or in an incubator at 37° for time intervals of four and 24 hours before they were separated into serum or plasma and frozen.

After the stated periods the tubes were centrifuged and the obtained samples of serum and plasma were stored at -80° until evaluation by enzyme-linked immunosorbent assay (ELISA).

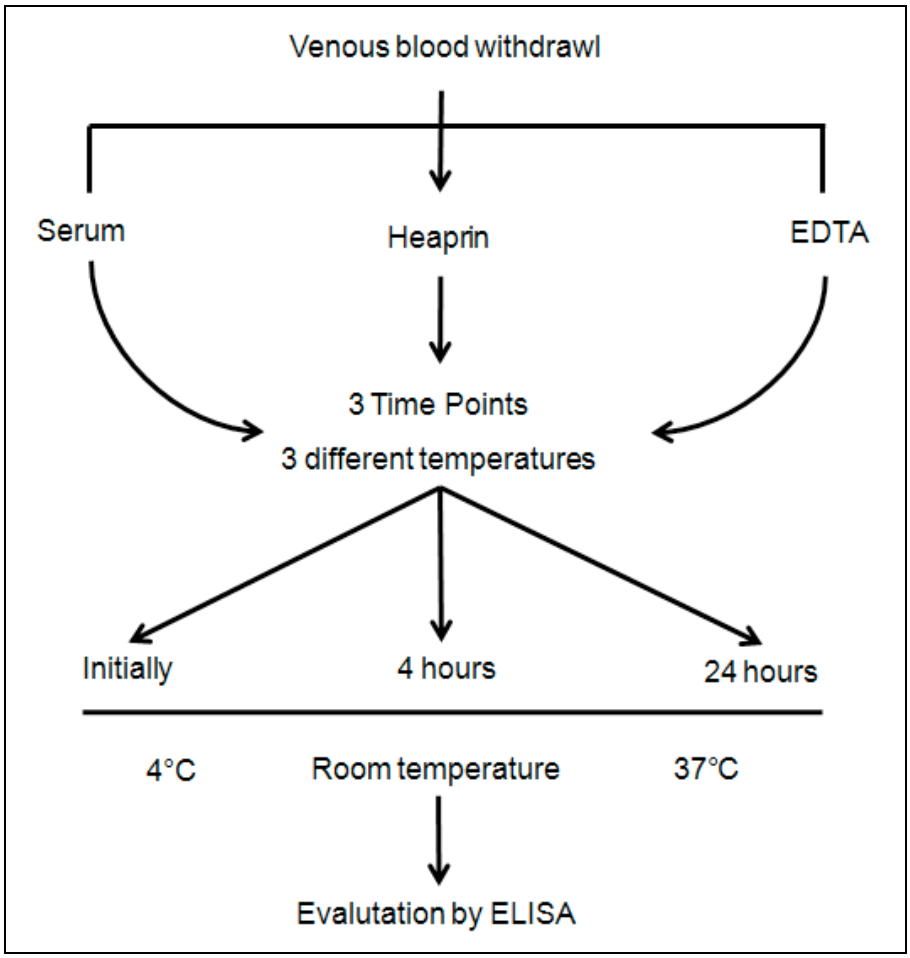

Figure 3.1 Whole Blood Experiment**:** Flow chart of study design and time points of Evaluation by ELISA: Venous blood was obtained by venipuncture using blood collection tubes for serum, heparinized plasma and EDTA plasma. To determine basic serum and plasma levels, selected tubes were centrifuged within 30 minutes after blood withdrawal and the obtained serum and plasma samples was be stored at -80°. The remaining tubes were placed at 4℃, at room temperature and at 37℃. After 4 and 24 hours one serum tube, one EDTA-plasma tube and one Heparin-plasma tube from 4℃, RT and 37℃, respectively was centrifuged and the aspirated serum and plasma sample was be stored at -80℃**.** Cytokines were measured by ELISA.

3.2 Separation of human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMC) were obtained from young healthy volunteers (n=12). Cells were separated by Ficoll-Paque (GE Healthcare Bio-Sciences AB, Sweden) density gradient centrifugation. In short, anticoagulated blood specimens were processed immediately after venipuncture, diluted 1:2 in Hanks balanced salt solution (HBBS, Lonza, Basel, Switzerland) and shifted carefully into 50 milliliter (ml) tubes containing Ficoll - Paque solution (GE Healthcare Bio - Sciences AB, Sweden). Tubes were centrifuged for 15 minutes at 800g at room temperature without brake and buffy coats with mononuclear cells were obtained. Cells were washed in HBSS and resuspended in fresh serum-free UltraCulture medium (Lonza,

Switzerland). Cell concentrations were determined on a Sysmex automated cell counter (Sysmex Inc., USA).

3.3 Cell Culture of human PBMC with autologous serum supplement

PBMC were seeded on 24 well plates (Corning Costar, USA) at a density of 1*10^6 cells per milliliter in UltraCulture Medium (Lonza, Switzerland). Initially thereafter, 200µl autologous serum was added to each well. The cell culture plates were incubated for 24 hours in an incubator with a humidified atmosphere. The addition of heparinized autologous plasma and fetal calf serum (PAA, Austria) served as additional entities in this experimental setting. For a second experiment, 1*10^6 cells per milliliter were incubated with increasing levels of autologous serum supplement (2.5%, 5%, 10% and 20%) in order to define dose dependency. After the incubation period, PBMC were separated from the cell culture supernatant by centrifugation at 400g for 9 minutes. The obtained specimens were kept frozen at -80°.

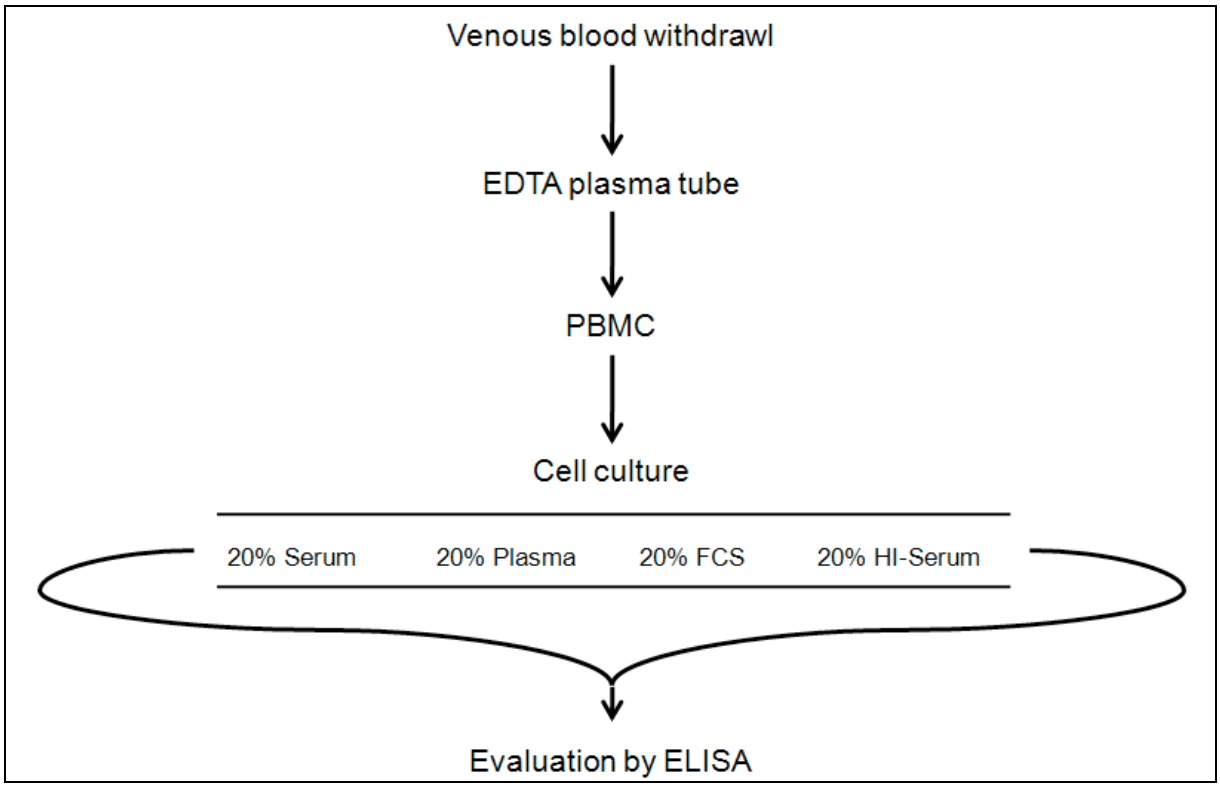

Figure 3.2 Stimulation assay: Flow chart of study design: Peripheral blood mononuclear cells were separated from EDTA tubes. They were seeded on 24 well plates and incubated with medium containing either 20% serum, or 20% plasma, or 20% FCS (fetal calf serum), or HI-serum (heat inactivated serum). After 24 hours at 37°C cell culture supernatant was separated and used for cytokine measurement by ELISA.

3.4 Stimulation of chemokine secretion by fibrin

Human PBMC were obtained as described above. Freeze-dried human fibrin (Sigma Aldrich, Austria) was resuspened in fresh UltraCulture Medium (Lonza, Switzerland) and aliquots were added in increasing concentrations (0.25µg, 0.5µg, 10µg, 50µg, 200µg and 500µg) to cell cultures of PBMC (cell density 1*10^6 per milliliter). PBMC cultured in medium without fibrin served as negative controls and cells incubated with increasing doses of autologous serum served as positive controls. After 24 hours of incubation supernatants were harvested and were stored until ELISA assays were conducted.

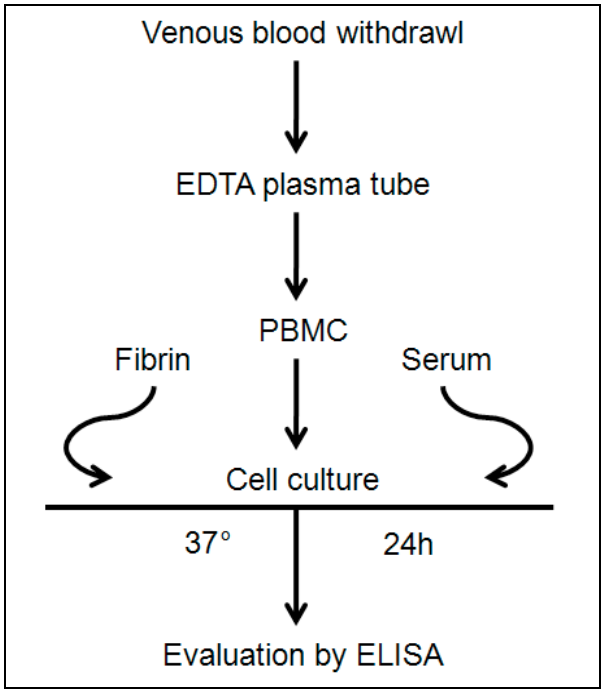

Figure 3.3 Fibrin stimulation assay: Flow chart of study design: Peripheral blood mononuclear cells were separated from EDTA tubes. They were seeded on 24 well plates and incubated with medium containing either increasing amount of fibrin, or increasing concentrations of serum . After 24 hours at 37℃ cell culture supernatant was separated and used for cytokine measurement.

Evaluation of serum, plasma and supernatant levels of selected cytokines by ELISA

Serum, plasma and cell culture supernatant levels of chemokines were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits for the quantification of IL-1β, IL-6, IL-8, GRO-α, ENA-78, GCP-2, MCP-1, RANTES, SDF-1, TNF-α, VEGF, TGF-β and IP-10 (Duoset; R&D Systems, USA) according to the manufacturer's protocol. In short, ninety-six–well microtiter plates were coated overnight at room temperature with the appropriate capture antibody. After blocking of plates, samples of serum, plasma, supernatants and standard protein were added

to the wells. After an incubation and a washing step, a biotin-labelled antibody was added to each well and incubated for 2 hours. Plates were washed and streptavidin-horseradishperoxidase was added for 30 minutes. Colour reaction was achieved using tetramethylbenzidine (TMB; Sigma Aldrich, USA) and was stopped by a sulphuric acid stop solution (Merck, Germany). Optical density values were measured at 450 nanometer on an ELISA plate reader (Victor3 Multilabel plate reader, PerkinElmer).

3.5 Proteome Profiler™ Array for determination of human angiogenesis-related proteins

Pooled samples (n=7) of baseline serum tubes and serum/ heparinized plasma/ EDTA plasma stored at 37°C for 24 hours were used to determine 55 angiogenesis related proteins with a Proteome Profiler™ Array (R&D System, USA). In short, samples were mixed with a cocktail of biotinylated detection antibody and then incubated with the array membranes pre-coated with capture antibodies. After a washing step, Streptavidin-HRP and chemiluminescent detection reagents were added sequentially. Positive signals seen after the exposure to an X-ray film for 1-10 minutes were identified by scanning the film on a transmission-mode scanner.

3.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, La Jolla, USA). All data are given as mean ± standard error of the mean (SEM). Based on high variability of cytokine levels in healthy subjects and our small sample size of ~7-12 participants we supposed that results are not normally distributed. The Friedman test, a non-parametric statistical test, was used to detect differences in serum or plasma tubes across multiple time points. Differences in the means of continuous measurements were tested by the Wilcoxon-signed-rank test. Due to the explorative character of this study no correction for multiple testing was performed[82]. A p value of <0.05 was considered to indicate statistical significance; all tests were two tailed.

3.7 Figures and illustrations

GraphPad Prism software (GraphPad Software, La Jolla, USA) was used to generate figures. For further processing, figures were converted into PowerPoint (Microsoft Corp., USA).

4 Results

4.1 Measurement of chemokine levels in serum, heparinized plasma and EDTA plasma

Samples of serum, heparinised plasma and EDTA plasma were obtained from seven healthy young volunteers by venipuncture using blood collection tubes routinely used in clinics (Vacuette®, Greiner BioOne, Austria). Some of the tubes were centrifuged shortly after blood withdrawal (30 minutes) in order to obtain basic values for cytokines. The remaining tubes were stored at three different temperature levels, at +4°C in a refrigerator, at room temperature (+24°C) and in an incubator at +37°C for four to 24 hours. The historical chemokine nomenclature is used in the following paragraphs. See table 2.1 for systematic nomenclature of chemokines.

4.1.1 Analysis of serum tubes

Serum concentrations of different cytokines are shown in table 4.1. Cytokine concentration seemed to be most stable when tubes were stored at 4°C, although GCP-2 levels were significantly elevated after an incubation period for four hours at 4°C.

A massive secretion of all chemokines and partially of cytokines (IL-8, GRO-α, MCP-1, IL-6, ENA-78, GCP-2, IL-1β, TNF-α, VEGF) was found in serum tubes when incubated at 37°C. A significant increase was measured for IL-8 and GCP-2 after only four hours. After 24 hours this resulted in a 44-fold increase for MCP-1, a 17-fold increase for GCP-2, a 22-fold increase for ENA-78, an 80-fold increase for GRO-α and even a 335-fold increase for IL-8 compared to basic levels.

A similar effect was seen for pro-inflammatory cytokines (IL-6, IL-1β) and growth factors (VEGF). Only TGF-β concentrations remained stable for all measured time points in serum tubes.

Serum (pg/ml)		4℃		24℃		37℃	
	0h	4h	24h	4h	24h	4h	24h
IL-8	66.3 ±11.9	71.9 ±11.0	290.8 ±215.1	68.5 ±11.0	356.2 ±122.9	594.0 ±192.7 *	22163.2 ±5343.4 **
GRO- α	64.4 ±24.9	11.4 ±6.3 *	28.5 ±9.8	10.0 ±4.6*	27.9 ±6.6	63.2 ±25.6	5180.2 ±849.3 *
ENA-78	3614.4 ±1402.8	3118.0 ±844.9	3507.4 ±1027.9	1881.2 ±845.1	2384.3 ±757.4	2359.4 ±1108.7	79789.8 ±10284.3 ***
GCP-2	517.1 ±38.2	766.5 ±57.1 **	879.8 ±87.6 **	711.3 ±64.4 *	706.5 ±92.9	689.8 ±57.1 **	8782.3 ±1653.9 **
MCP-1	43.7 ±41.8	1.9 ±1.9	20.9 ±19.7	8.3 ±6.1	11.6 ±6.4	18.1 ±6.3	1898.5 ±371.8 **
IL-6	88.0 ±20.2	84.9 ±27.8	64,9 ±32.2	56.2 ±24.6	48.18 ±26.6	54.4 ±31.7	6917.5 ±1743.9*
IL-1β	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	1156.5 ±415.7*
TNF- α	0.0 ±0.0	3.2 ±2.9	26.6 ±14.7	1.1 ±1.1	9.8 ±9.8	0.9 ±0.7	487.7 ±173.7*
TGF-β	643.2 ±19.4	756.4 ±204.6	967.0 ±249.5	1164.4 ±247.0	818.7 ±228.5	999.6 ±234.8	742.3 ±252.8
VEGF	733.5 ±523.8	177.1 ±78.9	80.5 ±80.5	755.3 ±376.2	395.2 ±209.3	257.8 ±95.8	436.1 ±136.0*

Table 4.1 Cytokine concentrations in serum tubes stored at different temperatures (4℃, 24℃, 37℃) for three different periods (0 hours, 4 hours, 24 hours). Basic serum levels are stated in column “0h”. Data are given as mean (± standard error of the mean). n=7

4.1.2 Analysis of heparinized plasma tubes

Minor to moderate difference of chemokines levels were seen between serum and plasma tubes that were process immediately. The increase in concentrations of detectable anylates in heparinized plasma was generally lower than in serum. Keeping blood samples at 4℃ before centrifugation and separation into plasma was effective to prevent an increase of measurable concentration of most anylates.

Thus, after four hours at 4℃ GCP-2 concentrations were significantly elevated and after 24 hours at 4℃ a 2 fold-increase was observed. Higher storage temperature and longer storage time leed to increased cytokine concentrations. Especially the measurable concentrations of IL-8, GRO-α, ENA-78 and MCP-1 increased notably after 24 hours at a storage temperature of 37℃. TNF-α, IL-1β and TGF-β were not detectable in most plasma samples.

Plasma Heparin (pg/ml)		4℃		24℃		37℃	
	0h	4h	24h	4h	24h	4h	24h
IL-8	65.4 ±12.9	65.8 ±10.9	77.4 ±18.8	68.8 ±12.8	165.1 ±32.0 *	357.2 ±183.9*	1973.9 ±562.9 *
GRO- α	28.5 ±18.4	2.8 ±1.4	23.1 ±10.2	21.3 ±6.4	22.8 ±9.4	11.5 ±8.4	222.1 ±85.4
ENA-78	448.1 ±346.5	2247.1 ±809.4	5171.1 ±1419.5 *	244.1 ±182.9	2100.8 ±977.9	665.5 ±581.2	9758.0 ±2717.8 *
GCP-2	324.7 ±45.3	500.6 ±65.3 **	736.9 ±67.6 ***	393.8 ±39.4	293.1 ±30.9	372.5 ±74.9	499.8 ±107.7
MCP-1	14.5 ±12.6	0.0 ±0.0	21.9 ±21.5	11.6 ±5.6	69.6 ±48.3	0.0 ±0.0	68.9 ±26.0 *
IL-6	59.4 ±25.5	41.6 ±28.7	27.0 ±8.9	33.9 ±13.0	56.4 ±20.7	86.3 ±46.0	142.0 ±71.3
IL-1β	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	7.1 ±4.6
TNF- α	0.0 ±0.0	17.9 ±8.7	14.8 ±13.9	0.0 ±0.0	0.0 ±0.0	37.5 ±37.5	0.6 ±0.6
TGF-β	0.0 ±0.0	37.4 ±18.0	366.0 ±131.6*	0.0 ±0.0	142.4 ±137.7	0.0 ±0.0	1.5 ±1.5
VEGF	701.1 ±578.9	460.9 ±376.5	284.5 ±162.7	210.0 ±143.0	251.3 ±221.2	320.8 ±118.5	165.8 ±128.1

Table 4.2 Cytokine concentrations in heparin tubes stored at different temperatures (4℃, 24℃, 37℃) for three different periods (0 hours, 4 hours, 24 hours). Basic plasma levels are stated in column “0h”. Data are given as mean (± standard error of the mean). n=7

4.1.3 Analysis of EDTA plasma tubes

Although the concentrations of anylates measured in EDTA plasma differed more from those in serum and heparinized plasma when being analyzed initially, EDTA plasma levels showed the least divergence after 24 hours, even at temperatures above 24°C. IL-8, GRO-α, ENA-78, MCP-1 and all cytokine concentrations were stable in EDTA plasma for all test samples. GCP-2 was the only protein showing significant alterations. TNF-α, IL-1β and TGF-β were not detectable in most plasma samples.

Baseline levels of TGF-β were not detectable in heparinized plasma samples, whereas baseline levels of TGF-β in serum tubes were 6-times higher than in EDTA plasma (serum: 643.2 ±19.4 vs. EDTA plasma 92.1 ±82.5). A similar or greater difference between the serum and plasma levels was observed at all other time points.

Plasma EDTA (pg/ml)		4℃		24℃		37℃	
	0h	4h	24h	4h	24h	4h	24h
IL-8	97.7 ±21.4	233.4 ±144.6	74.1 ±17.8	110.9 ±27.8	82.1 ±22.3	67.8 ±12.7	118.9 ±22.7
GRO- α	32.6 ±23.3	7.4 ±2.7	11.9 ±3.7	17.6 ±9.4	15.9 ±8.3	13.5 ±13.3	31.9 ±9.5
ENA-78	963.9 ±771.7	343.6 ±301.1	954.5 ±413.5	685.6 ±350.3	1620.9 ±594.5	58.5 ±58.5	1421.0 ±578.5
GCP-2	315.9 ±54.2	248.9 ±38.3 *	368.7 ±54.5	119.7 ±21.2 **	200.3 ±26.4 **	156.0 ±23.0 *	306.5 ±19.2
MCP-1	23.7 ±20.6	0.4 ±0.4	59.4 ±30.5	35.2 ±14.5	24.1 ±10.2	9.7 ±4.6	18.3 ±4.3
IL-6	93.8 ±48.3	37.9 ±11.5	68.1 ±35.2	78.0 ±25.4	48.5 ±23.6	56.5 ±28.7	81.5 ±32.4
IL-1β	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	7.6 ±6.9
TNF- α	8.5 ±7.7	0.0 ±0.0	17.4 ±11.6	7.7 ±4.0	0.0 ±0.0	13.9 ±13.9	5.1 ±4.9
TGF-β	92.1 ±82.5	39.5 ±37.5	83.0 ±60.0	0.0 ±0.0	40.9 ±30.0	0.0 ±0.0	100.5 ±52.6
VEGF	619.9 ±318.5	53.2 ±29.4	143.0 ±81.2	320.5 ±203.1	131.8 ±66.3	111.6 ±102.2	219.4 ±108.6

Table 4.3 Cytokine concentrations in EDTA tubes stored at different temperatures (4℃, 24℃, 37℃) for three different periods (0 hours, 4 hours, 24 hours). Basic plasma levels are stated in column “0h”. Data are given as mean (± standard error of the mean). n=7

4.2 Angiogenesis array

55 proteins associated with neo-angiogenesis were determined by a membrane array. Results are shown in figure 4.1 and 4.2. After incubation of blood samples at 37°C for 24 hours prior to centrifugation (n=7), several proteins are increased in serum, heparinized plasma and EDTA plasma. In EDTA plasma higher amounts of Serpin E1, MMP-9, TIMP-1and PDGF-AA Angiogenin-1 were detected compared to serum processed immediately after venipuncture.

In heparinized plasma Serpin E1, TIMP-1 and PDGF-AA were elevated compared to control serum. IL-8 concentrations, which were not detectable in control serum, were massively detectable in heparinized plasma.

The following cytokines were only detectable in serum samples stored 24 hours at 37°C: MIP-1α, epidermal growth factor (EGF), IL-1β and MCP-1.

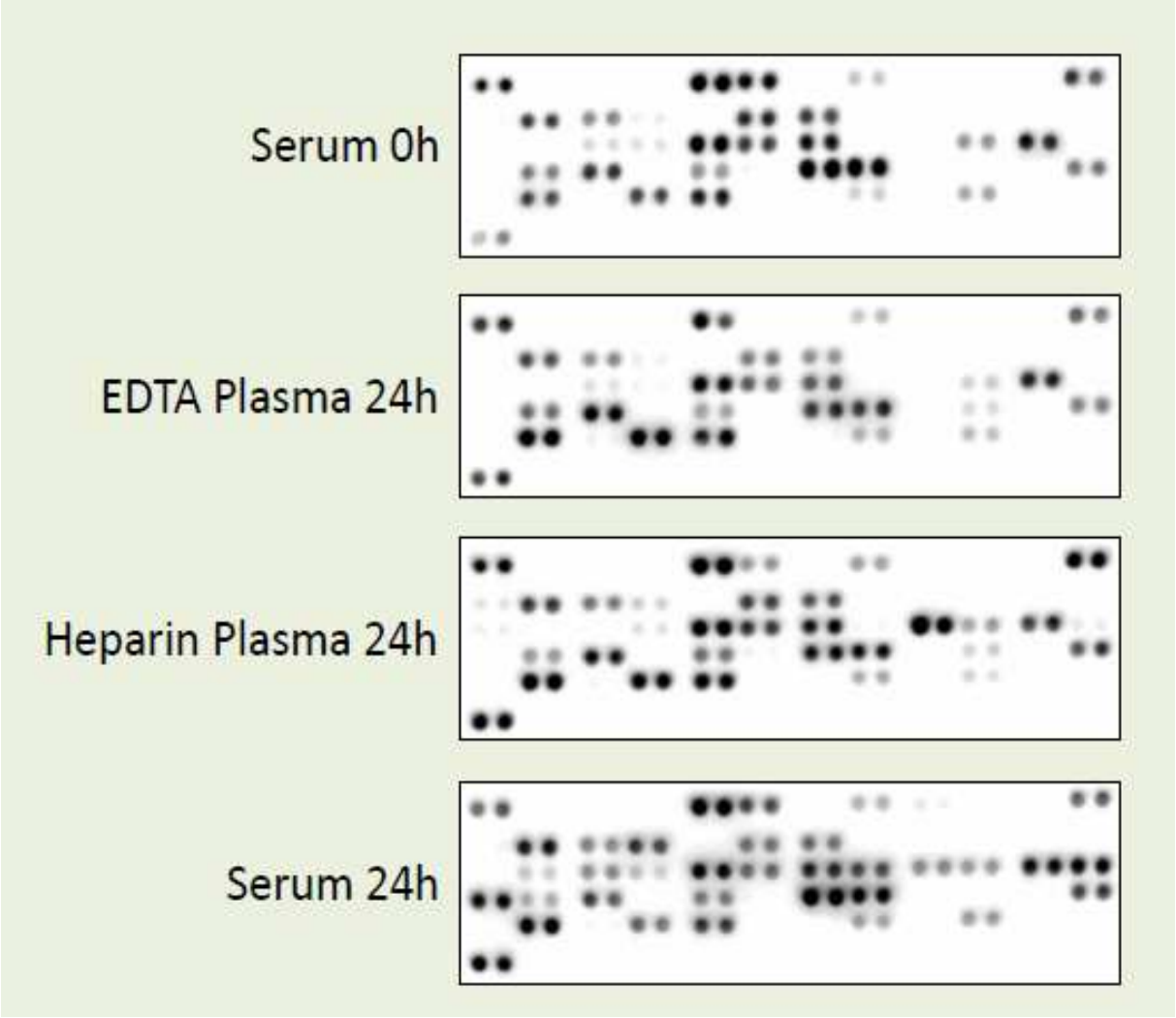

Figure 4.1 Angiogenesis array: semi-quantitative measurement of 55 proteins after blood withdrawal (Serum 0h), and after a storage of 24 hours at 37°C (EDTA Plasma, Heparin Plasma, Serum). Pooled samples from 7 subjects.

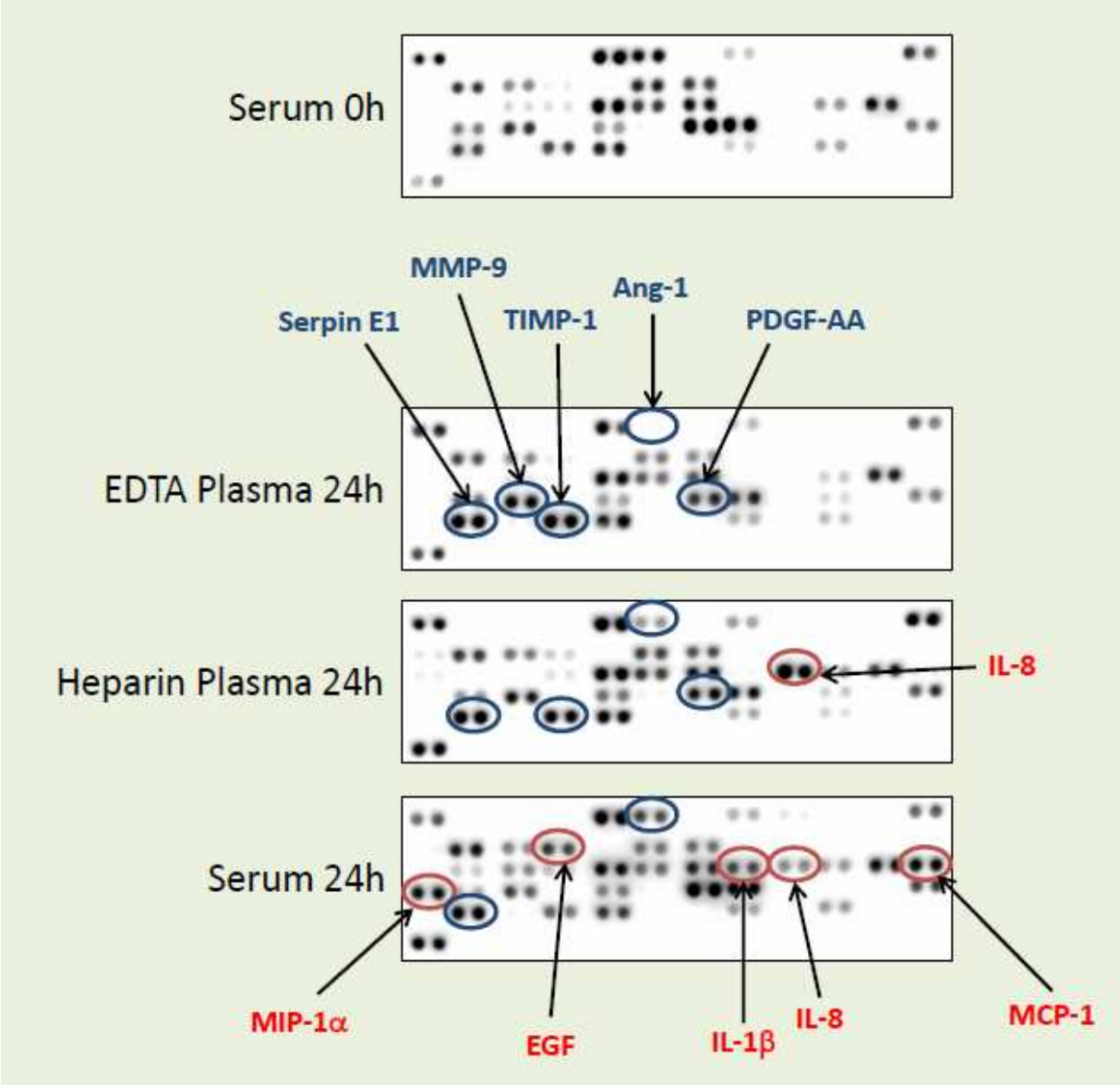

Figure 4.2 Angiogenesis array: Proteins which were detected in higher concentration than in serum at 0h were highlighted in blue. Red highlighted proteins were not detectable at basic serum samples.

4.3 Induction of chemokine secretion of human peripheral blood mononuclear cells by autologous serum

Based on the observation that especially chemokines were massively secreted in serum tubes when being incubated we sought to investigate the nature of serum induced release of chemokines and other factors from human white blood cells.

A high increase of IL-8, GRO-α, ENA-78, GCP-2, MCP-1 and MIP-1β was observed when human PBMCs were incubated of with 20% autologous serum. This increment of chemokine secretion was weaker or less detectable when 20% autologous heparinized plasma was added. The incubation with 20% fetal calf serum (FCS)

resulted in a comparable induction of chemokine secretion compared with heparinized plasma. As FCS is often routinely heat-inactivated (by being incubated in a water bath at 56°C in order to inactivate complement and coagulation factors) and filtered through 0,1µm membranes we sought to investigate commercially available FCS with freshly prepared autologous serum that underwent a heat-inactivation protocol.

Supernatants obtained from PBMC incubated with FCS showed only a marginal increase of chemokine secretion compared to base line values. Heat inactivated serum from humans evidenced a comparable potential to serum to induce chemokine secretion. This secretion pattern was evident for all tested CXCR2 (IL-8, GRO-α, ENA-78, GCP-2) and CCR2 (MCP-1) associated chemokines but not for CXCR3 and 4 related chemokines (SDF-1 and IP-10) (see table 4.4).

The pro-inflammatory chemokines IL-6 and TNF-α were also found in higher concentrations after incubation human PMBCs with autologous serum compared to incubation with heparinized plasma. TGF-β and VEGF did not show an increment when cells were incubated together with 20% autologous serum or with 20% heparinized plasma.

(pg/ml)	Medium	plus 20% Serum	plus 20% Plasma	plus 20% FCS	plus 20% HI-Serum
IL-8	373.4 ±81.4	12065.5 ±1295.9 ***	833.7 ±369.8	616.2 ±136.1	4448.5 ±795.4 ***
GRO-α	61.9 ±21.9	4988.9 ±241.9 ***	394.1 ±221.7	136.9 ±44.4	3675.2 ±722.1 ***
ENA-78	438.2 ±210.2	23546.8 ±1585.3 ***	1851.2 ±1067.2	1099.7 ±297.6	10767.5 ±2118.2 ***
GCP-2	59.1 ±18.5	1204.4 ±226.6 ***	149.6 ±78.9	46.9 ±20.4	688.1 ±157.4 ***
MCP-1	682.1 ±190.8	23457.6 ±980.2 ***	6215.6 ±2335.5 **	2116.3 ±564.7 *	15141.5 ±3040.5 ***
MIP-1β	30.5 ±13.9	936.6 ±140.9 ***	130.6 ±44.9 *	23.9 ±5.6	224.5 ±77.0 **
RANTES	4764.8 ±634.4	9116.3 ±715.6 ***	3161.7 ±178.0	6381.0 ±1304.0	10033.9 ±983.7 ***
SDF-1	37.0 ±9.8	0.0 ±0.0 **	0.0 ±0.0 **	9.9 ±6.1	59.9 ±19.4
IP-10	19.6 ±10.5	7.8 ±6.6	931.1 ±347.5 **	185.3 ±135.4	144.9 ±97.1
IL-6	0.0 ±0.0	613.1 ±108.1***	3.6 ±2.2	0.2 ±0.2	155.9 ±127.7*
TNF-α	1.2 ±0.8	83.3 ±33.0*	0.0 ±0.0	0.0 ±0.0	1.2 ±0.9
VEGF	61.5 ±9.4	21.6 ±5.9*	0.0 ±0.0*	31.7 ±5.1*	41.1 ±9.9*
TGF-β	17.4 ± 2.9	41.4 ± 4.6***	10.9 ± 1.9	17.9 ±3.0***	44.4 ±5.4

Table 4.4 Cytokine concentrations measured in cell culture supernatant from 1*10^6 PBMCs per milliliter. PBMCs were seeded with medium, or medium containing 20% autologous serum, or 20% autologous plasma, or 20% FCS (fetal calf serum), or 20% HI-serum (heat inactivated serum). Data are given as mean (± standard error of the mean). n=8-12

4.3.1 Dose dependency of serum induced chemokines secretion

When autologous serum was added in increasing doses, a significant induction of IL-8 secretion was measured, even at concentrations as low as 2,5% (1620.1 ±472.1 pg/mL) compared to baseline concentrations (368.5 ±170.9pg/mL). IL-8 secretion was induced in a dose depending fashion to 4777.8 ±1243.1 pg/mL when 5% serum were addend, to 6635.46 ±1886.8 pg/mL when 10% serum were added and slightly decreased again to 4134.3 ±1288.6 with 20% serum supplement (n=8) (see figure 4.3). A similar dose dependency was measured for the CXC2R binding chemokines ENA-78 and GRO-α.

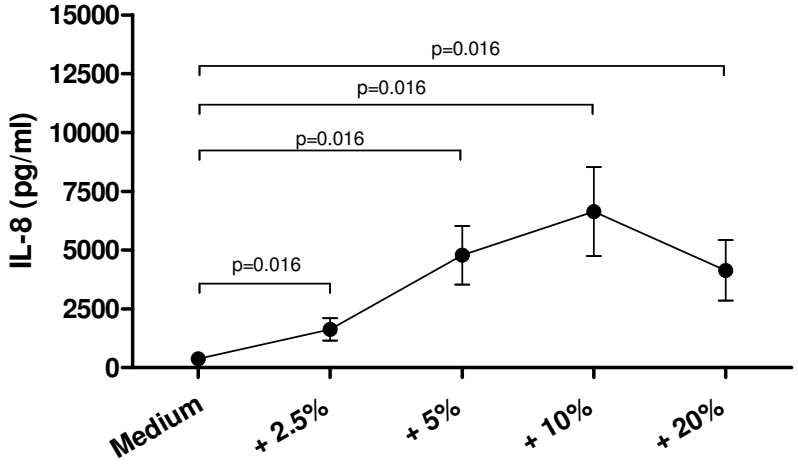

Figure 4.3 Incubation of PBMC with increasing concentrations of autologous serum induct an increase of IL-8 secretion (mean±SEM) n=8.

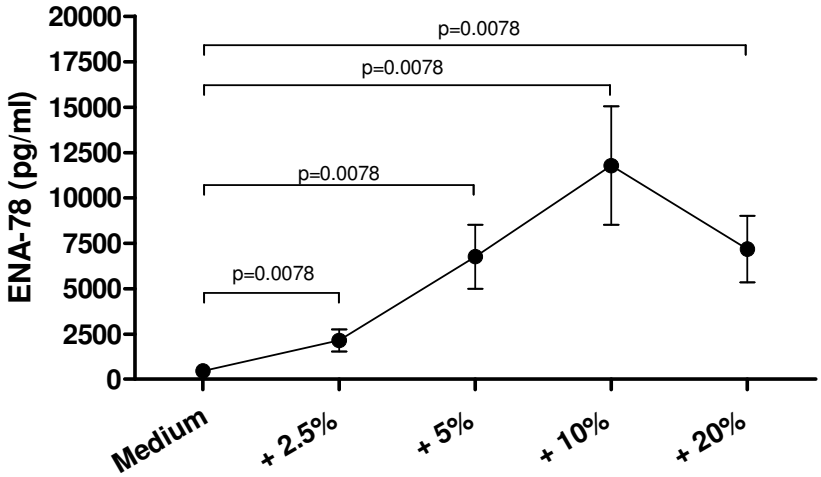

Figure 4.4 Incubation of PBMC with increasing concentrations of autologous serum induct an increase of ENA-78 secretion (mean±SEM) n=8.

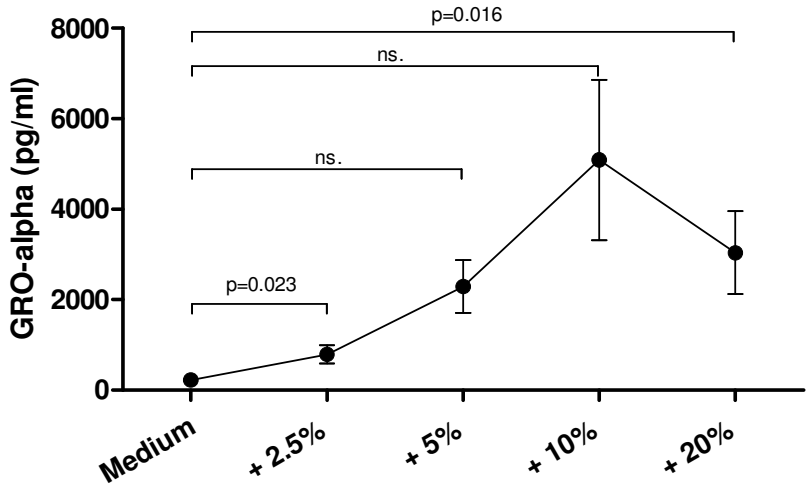

Figure 4.5 Incubation of PBMC with increasing concentrations of autologous serum induct an increase of IL-8 secretion (mean±SEM) n=8.

Autologous serum supplement did not influence IL-1β nor IL-1RA levels (see figure 4.6).

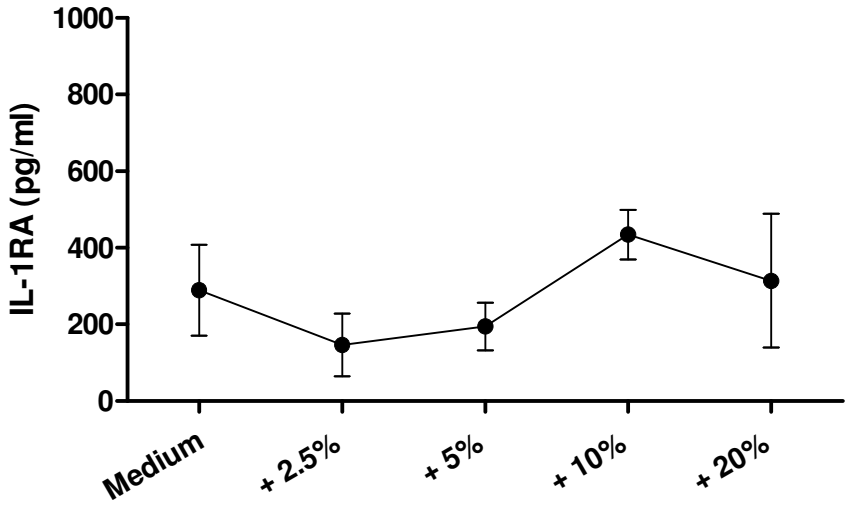

Figure 4.6 Incubation of PBMC with increasing concentrations of autologous serum do not induct an increase of IL-1RA secretion (mean±SEM) n=8.

4.4 Cytokine secretion triggered by fibrin

Fibrin was observed to induce a massive cytokine release in a dose dependent manner (see figure 4.7-10). Low amounts of fibrin (0.25 µg – up to 0.5 µg fibrin) that were added to PBMCs leed to a weak increase. A highly significant induction of all measured cytokines in this experiment was examined when 50 µg or more of fibrin were cell culture wells ($p<0.001$).

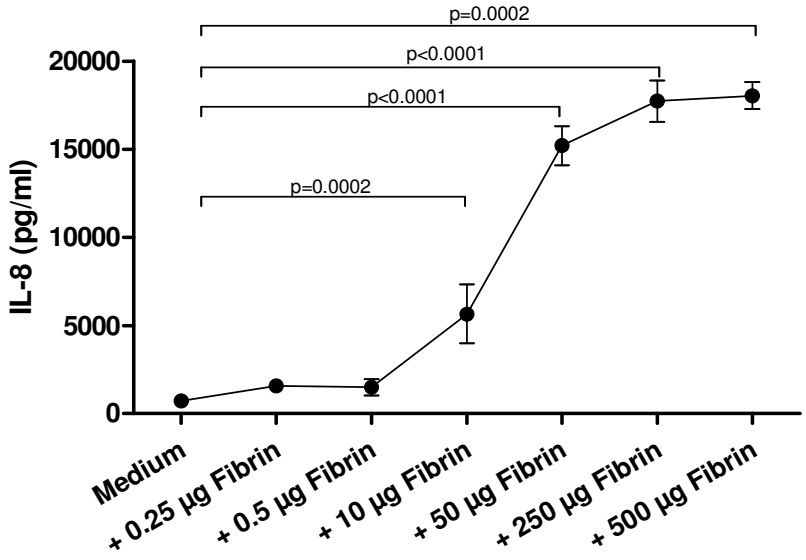

Figure 4.7 Fibrin triggers IL-8 secretion of PBMC dose dependent. (mean±SEM) n=8.

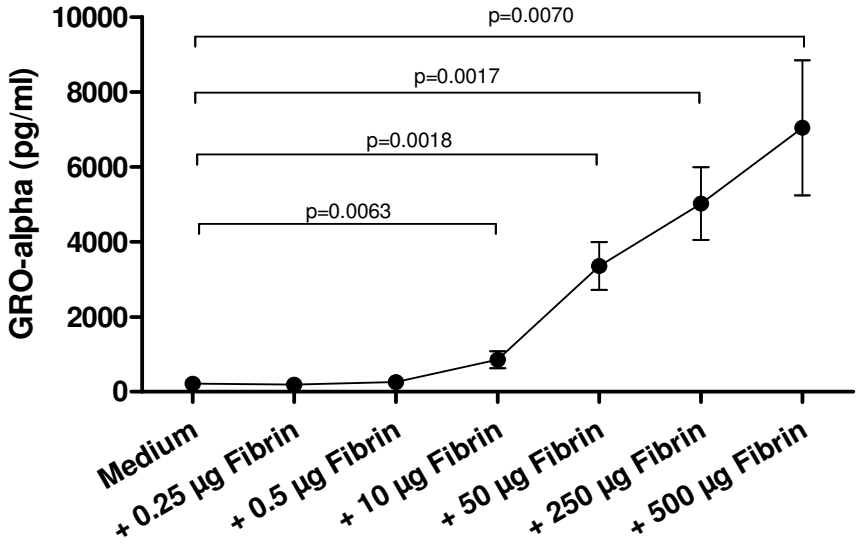

Figure 4.8 Fibrin triggers GRO-α secretion of PBMC dose dependent. (mean±SEM) n=8.

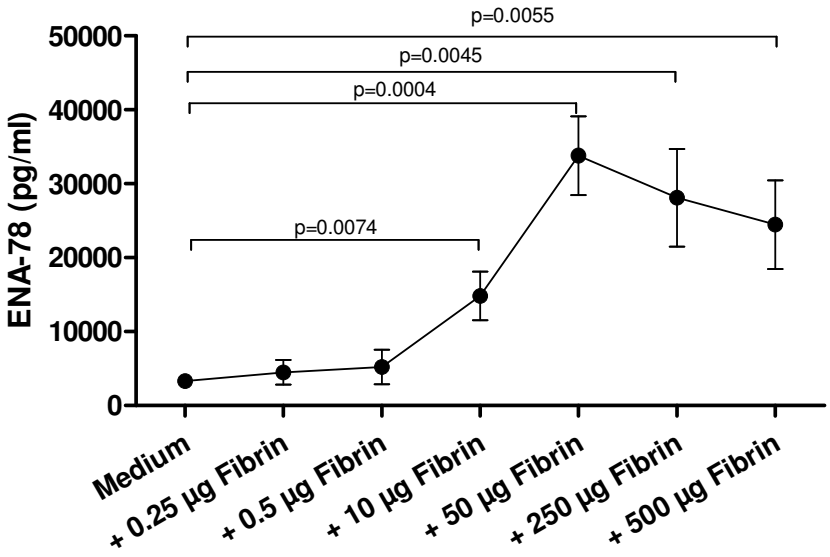

Figure 4.9 Fibrin triggers ENA-78 secretion of PBMC dose dependent. (mean±SEM) n=8.

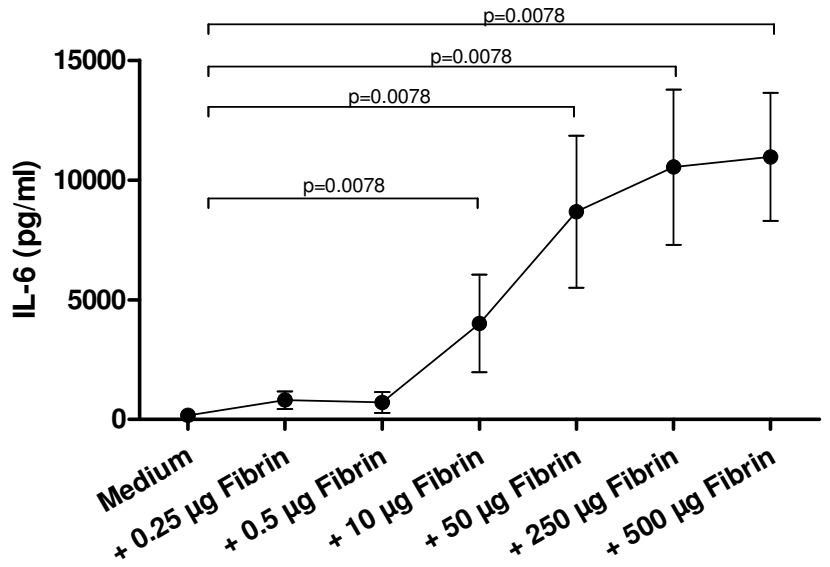

Figure 4.10 Fibrin triggers IL-6secretion of PBMC dose dependent. (mean±SEM) n=8.

5 Discussion

In the present study we evaluated the stability of cytokines and chemokines in blood samples of healthy subjects. In order to prove our data obtained from whole blood experiments, we demonstrated in an *in vitro* experiment that serum components induce an enhanced secretion of cytokines and chemokines of PBMCs. Based on this finding we assumed that coagulation products trigger the protein release. In a further *in vitro* assay we demonstrated that fibrin had the ability to enhance cytokine and chemokine secretion.

Since the analysis of cytokine concentrations in serum or plasma of patients has become an important research issue in several diseases, work has to be undertaken to evaluate the stability of these mediators in blood samples. Hence, we sought to investigate the effect of sample storage period and storage temperature on anylates concentration in serum and plasma.

We measured the concentration of cytokines in serum and plasma, after exposure of blood to different conditions before centrifugation (time periods varying from 30 minutes to 24 hours). Furthermore, we evaluated the influence of different storage temperatures (4°C, RT, 37°C) hypothesizing that the storage temperature serves as an important influence factor for many cellular reactions such as paracrine response[65, 68].

We were able to show that both activation of coagulation cascade in serum tubes and temperature above 24°C (room temperature) massively triggers chemokine release from cellular compartments. Therefore, rapid separation of serum and plasma from cellular components and freezing of samples is essential to preserve reliability of markers in samples for later analysis. If it is not possible to separate cellular components in samples immediately after blood draw, as might be expected, blood storage at 4°C seems to be the best way to achieve baseline chemokine concentrations.

However, it has to be noted that even a storage at 4°C for four hours can significantly alter cytokine concentrations. After 24 hours of storage most chemokine concentrations were significantly higher compared to baseline concentrations.

This study shows that cytokine concentrations measured in EDTA plasma remain the most stable and seems to be suitable for stability reasons. This could be explained by the fact EDTA is a chelating agent which binds metal ions such as Ca^{2+} and F^{3+}. Ca^{2+} is a vital co-factor for cytokine release, known to effect cellular functions, including secretion of IL-6, chemo attraction and apoptosis[83, 84]. However, in base line specimens EDTA plasma evidenced the highest variability between samples. This might be caused by interferences of EDTA with ELISA systems.

In agreement with some studies[65], but not with others[68], a significant relationship between IL-6 and storage time and storage temperature was found. We claim that IL-6 is rather produced *de novo* than released from stored cellular pools, because of its time dependent increase.

Previous studies compared cytokine values in blood spiked with cytokines prior to separation into serum or plasma and stored this samples for up to 20 days at different temperatures[76, 79, 80]. The latter examined decreased TNF-α concentrations in samples kept at room temperature compared to samples stored at 4°C and 70°C. Thavasu *et al.* performed a study on whole blood spiked with TNF-α, IL-6, IL-1β, IL-1α, Interferon α, and Interferon gamma stored at 4°C or room temperature. The recovery of all anylates except IL-1α was lower after a storage at room temperature compared to 4°C. Furthermore, the measurable concentrations of serum samples spiked with cytokines were by trend lower than concentration recovered for plasma samples, which is in contrast to the increase of cytokines in whole blood samples, as we found.

It appears that exogen added cytokines to whole blood samples are absorbed by blood cells or are bound to cellular components and therefore are not accessible for measurement with immunoassays. Marie *et al.* examined that up 96% of exogenous added IL-8 is associated with blood cells and became measureable after cell lysis[85]. Recovery of IL-8 added to whole EDTA plasma samples was discovered by Reinsberg *et al.* This group reported that <35% of added IL-8 to EDTA whole blood samples could be recovered, whereas the recovery from plasma lysate was 104% indicating that cells are responsible for IL-8 sequestration[86].

In order to prove our results seen in whole blood samples, we tried to reproduce the results in cell cultures of human PBMC which were supplemented with serum, plasma, FCS or heat inactivated serum. Similarly as in the whole blood incubation experiment, PBMC cell cultures supplemented with autologous serum evidenced a significant induction of chemokine secretion. This magnitude of cytokine induction was not apparent when plasma or FCS was added.

Heat inactivated serum exposed to be a comparable trigger to serum for chemokine secretion. We evaluated the influence of heat inactivated serum in order to determine the influence of immunological factors on chemokine secretion. Heat inactivated serum is used to inactivate immune factors, particularly serum complement to preserve integrity of immunoassays[87]. Complement factors are known to stimulate cytokine release in humans[88-90]. We were able to show that heat activated serum had almost the same potential to induce cytokine production of human PBMC as non heat inactivated serum. Therefore, we conclude that complement factors were not responsible for the massive secretion of proteins in serum tubes in our study.

Based on these results we speculated that a high molecular coagulation product such as fibrin might be accountable for induction of chemokine secretion. As FCS undergoes many filtration steps, proteins of higher molecular structures are removed, therefore it seems likely that FCS failed to produce a comparable cytokine secretion pattern.

Fibrin induces synthesis of chemokines and pro-inflammatory cytokines in human PBMC.

To further verify our hypothesis, we incubated human PBMC with increasing doses of fibrin. In order to reproduce an *in vivo* situation we used Fibrin concentration found in serum[91] respectively higher fibrin concentrations used in *in vitro* experiments[92]. According to previous studies on PBMCs[92], macrophages[93], human synovial fibroblasts[94] and human vascular endothelial cells[95, 96] the supplement of fibrin induces a highly significant IL-8 induction.

During the coagulation process, thrombin cleaves fibrinogen releasing the fibrinopeptides A and B and fibrin. The fibrin monomers further polymerize and are

finally stabilized by coagulation factor XIIIa catalyzing the formation of isopeptide bonds between the gamma chains of two fibrin molecules [97].

The exact mechanism of the cross-talk between coagulation and inflammation is still not elucidated. Several reports in the literature describe the linkage between inflammation and coagulation products[91, 98, 99], whereas fibrinogen as well as fibrin have a pro-inflammatory capacity[92, 97].

Pro-angiogenic capacity of serum tube

Based on previous reports, indicating that 20% serum supplement in cell based therapies has positive effects on cell viability[100], neovascularization capacity and migration capacity of bone marrow cells[100], we measured 55 pro-angiogenic proteins in 4 pooled (n=7) samples. Therefore a commercially available membrane array (R&D Systems) for detection of pro-angiogenic proteins was used.

We detected elevated amounts of pro-angiogenic proteins in serum samples as well as in heparin plasma and EDTA plasma samples when tubes were stored at 37°C for 24 hours compared to tubes centrifuged immediately after blood withdrawal. The most pro-angiogenic proteins were detected in samples from serum tubes.

These proteins might be responsible for the enhanced migratory capacity seen of bone marrow cells after a 12 hour incubation with 20% serum compared to the incubation with 20% heparinized plasma[100].

5.1 Limitations

This study has some limitations worth pointing out. The sample size was low (4 to 8 participants per assay), and does not reflect hospitalized patient in age and illness.

Due to our limited PBMC purification process, a contamination of PBMC cultures with erythrocytes and platelets has to be assumed. Platelets store high amounts of chemokines such as IL-8, TGF-β, which are released after platelet activation[70, 101]. However, the time dependent increase and the high amount of measured anylates suggest rather a *de novo* production than only a release of pre-assembled cytokines.

5.2 Clinical implications

Based on our findings, we suggest following instruction in handling blood samples for chemokine analysis

- immediate processing of plasma and serum is essential
- non-centrifuged samples should be stored at 4°C
- comparison between serum and plasma concentrations have to be interpreted critically
- EDTA plasma seems to be most suitable for stability reasons, whereas EDTA can interfere with the test system.

In conclusion, we were able to show in this study that cytokine levels are highly susceptible to sample handling and processing prior to separation into serum or plasma. Cytokine levels remain more stable in plasma than in serum and the accumulation of cytokines in serum samples in part is regulated by coagulation products.

6 References

1. Tedgui A, Mallat Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev* 2006; 86(2):515-81.
2. Kronfol Z. Cytokines And Mental Health. *In* Kronfol Z, ed., Vol. 1. Berlin: Kluwer Academic Publishers, 2003.
3. Beutler BA, Milsark IW, Cerami A. Cachectin/tumor necrosis factor: production, distribution, and metabolic fate in vivo. *J Immunol* 1985; 135(6):3972-7.
4. Roumen RM, Hendriks T, van der Ven-Jongekrijg J, et al. Cytokine patterns in patients after major vascular surgery, hemorrhagic shock, and severe blunt trauma. Relation with subsequent adult respiratory distress syndrome and multiple organ failure. *Ann Surg* 1993; 218(6):769-76.
5. Biffl WL, Moore EE, Moore FA, et al. Interleukin-6 in the injured patient. Marker of injury or mediator of inflammation? *Ann Surg* 1996; 224(5):647-64.
6. Roth GA, Zimmermann M, Lubsczyk BA, et al. Up-regulation of interleukin 33 and soluble ST2 serum levels in liver failure. *J Surg Res* 2010; 163(2):e79-83.
7. Brunner M, Krenn C, Roth G, et al. Increased levels of soluble ST2 protein and IgG1 production in patients with sepsis and trauma. *Intensive Care Med* 2004; 30(7):1468-73.
8. Ploder M, Pelinka L, Schmuckenschlager C, et al. Lipopolysaccharide-induced tumor necrosis factor alpha production and not monocyte human leukocyte antigen-DR expression is correlated with survival in septic trauma patients. *Shock* 2006; 25(2):129-34.
9. Hacker S, Lambers C, Pollreisz A, et al. Increased soluble serum markers caspase-cleaved cytokeratin-18, histones, and ST2 indicate apoptotic turnover and chronic immune response in COPD. *J Clin Lab Anal* 2009; 23(6):372-9.
10. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. *Nat Rev Immunol* 2007; 7(6):429-42.
11. Rahman A, Isenberg DA. Systemic lupus erythematosus. *N Engl J Med* 2008; 358(9):929-39.
12. Mantovani A, Allavena P, Sica A, et al. Cancer-related inflammation. *Nature* 2008; 454(7203):436-44.
13. Belperio JA, Keane MP, Arenberg DA, et al. CXC chemokines in angiogenesis. *J Leukoc Biol* 2000; 68(1):1-8.
14. Strieter RM, Polverini PJ, Kunkel SL, et al. The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *J Biol Chem* 1995; 270(45):27348-57.
15. Luster AD. Chemokines--chemotactic cytokines that mediate inflammation. *N Engl J Med* 1998; 338(7):436-45.
16. Ponath PD, Qin S, Ringler DJ, et al. Cloning of the human eosinophil chemoattractant, eotaxin. Expression, receptor binding, and functional properties suggest a mechanism for the selective recruitment of eosinophils. *J Clin Invest* 1996; 97(3):604-12.
17. Balestrieri ML, Balestrieri A, Mancini FP, et al. Understanding the immunoangiostatic CXC chemokine network. *Cardiovasc Res* 2008; 78(2):250-6.

18. Rajagopalan L, Rajarathnam K. Structural basis of chemokine receptor function--a model for binding affinity and ligand selectivity. *Biosci Rep* 2006; 26(5):325-39.
19. Colobran R, Pujol-Borrell R, Armengol MP, et al. The chemokine network. I. How the genomic organization of chemokines contains clues for deciphering their functional complexity. *Clin Exp Immunol* 2007; 148(2):208-17.
20. Bernardini G, Hedrick J, Sozzani S, et al. Identification of the CC chemokines TARC and macrophage inflammatory protein-1 beta as novel functional ligands for the CCR8 receptor. *Eur J Immunol* 1998; 28(2):582-8.
21. Le Y, Zhou Y, Iribarren P, et al. Chemokines and chemokine receptors: their manifold roles in homeostasis and disease. *Cell Mol Immunol* 2004; 1(2):95-104.
22. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. *Immunity* 2000; 12(2):121-7.
23. Umehara H, Bloom ET, Okazaki T, et al. Fractalkine in vascular biology: from basic research to clinical disease. *Arterioscler Thromb Vasc Biol* 2004; 24(1):34-40.
24. Premack BA, Schall TJ. Chemokine receptors: gateways to inflammation and infection. *Nat Med* 1996; 2(11):1174-8.
25. Murphy PM, Tiffany HL. Cloning of complementary DNA encoding a functional human interleukin-8 receptor. Science. 1991. 253: 1280-1283. *J Immunol* 2009; 183(5):2898-901.
26. Lazennec G, Richmond A. Chemokines and chemokine receptors: new insights into cancer-related inflammation. *Trends Mol Med* 2010; 16(3):133-44.
27. Rosenfeld L. A golden age of clinical chemistry: 1948-1960. *Clin Chem* 2000; 46(10):1705-14.
28. Diagnostics B. Tube Guide 2010. Available at: http://www.bd.com/vacutainer/pdfs/plus_plastic_tubes_wallchart_tubeguide_VS5229.pdf.
29. Wilde C. Labatory Management. *In* Wild D, ed. The Immunoassy Handbook, Vol. Third Edition. Netherlands: Elsevier L.td, 2005. pp. 443 to 455.
30. D.J. E. Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture; Approved Standard - Sixth Edition. Vol. 27. Wayne, Pennsylvania USA: Clinical Laboratory Standards Institue, 2003.
31. Westwood FCHOMR. Practical Immunology. *In* Sons JW, ed. Oxford: Blackwell Publishing Company, 2002.
32. Zhou X, Fragala MS, McElhaney JE, et al. Conceptual and methodological issues relevant to cytokine and inflammatory marker measurements in clinical research. *Curr Opin Clin Nutr Metab Care* 2010; 13(5):541-7.
33. Jacques HRVD. Cytokines im Human Health. *In* A. T, ed. Immunotoxicology, Pathology and Therapeutic Applications. Totowa: Humana Press Inc., 2007.
34. Leng SX, McElhaney JE, Walston JD, et al. ELISA and multiplex technologies for cytokine measurement in inflammation and aging research. *J Gerontol A Biol Sci Med Sci* 2008; 63(8):879-84.
35. Stefura WP, Campbell JD, Douville R, et al. Ultrasensitive ELISA for measurement of human cytokine responses in primary culture. *Methods Mol Med* 2008; 138:107-19.
36. Tarrant JM. Blood cytokines as biomarkers of in vivo toxicity in preclinical safety assessment: considerations for their use. *Toxicol Sci* 2010; 117(1):4-16.

37. Ledur A, Fitting C, David B, et al. Variable estimates of cytokine levels produced by commercial ELISA kits: results using international cytokine standards. *J Immunol Methods* 1995; 186(2):171-9.
38. Vignali DA. Multiplexed particle-based flow cytometric assays. *J Immunol Methods* 2000; 243(1-2):243-55.
39. Multimetrix. Luminex-Analyzer 2011. Available at: http://www.multimetrix.com/technologie/testprinzip.html. 2011.
40. Elshal MF, McCoy JP. Multiplex bead array assays: performance evaluation and comparison of sensitivity to ELISA. *Methods* 2006; 38(4):317-23.
41. Vazquez-Martin A, Colomer R, Menendez JA. Protein array technology to detect HER2 (erbB-2)-induced 'cytokine signature' in breast cancer. *Eur J Cancer* 2007; 43(7):1117-24.
42. Pohl D, Andrys C, Borska L, et al. CC and CXC chemokines patterns in psoriasis determined by protein array method were influenced by Goeckerman's therapy. *Acta Medica (Hradec Kralove)* 2009; 52(1):9-13.
43. Middleton RK, Lloyd GM, Bown MJ, et al. The pro-inflammatory and chemotactic cytokine microenvironment of the abdominal aortic aneurysm wall: a protein array study. *J Vasc Surg* 2007; 45(3):574-80.
44. Kverka M, Burianova J, Lodinova-Zadnikova R, et al. Cytokine profiling in human colostrum and milk by protein array. *Clin Chem* 2007; 53(5):955-62.
45. Joos TO, Berger H. The long and difficult road to the diagnostic market: protein microarrays. *Drug Discov Today* 2006; 11(21-22):959-61.
46. Joos TO, Stoll D, Templin MF. Miniaturised multiplexed immunoassays. *Curr Opin Chem Biol* 2002; 6(1):76-80.
47. Templin MF, Stoll D, Schrenk M, et al. Protein microarray technology. *Trends Biotechnol* 2002; 20(4):160-6.
48. Hartmann M, Roeraade J, Stoll D, et al. Protein microarrays for diagnostic assays. *Anal Bioanal Chem* 2009; 393(5):1407-16.
49. Sachdeva N, Asthana D. Cytokine quantitation: technologies and applications. *Front Biosci* 2007; 12:4682-95.
50. House RV. Cytokine measurement techniques for assessing hypersensitivity. *Toxicology* 2001; 158(1-2):51-8.
51. van Assen S, de Haan A, Holvast A, et al. Cell-mediated immune responses to inactivated trivalent influenza-vaccination are decreased in patients with common variable immunodeficiency. *Clin Immunol* 2011; 141(2):161-8.
52. Lalvani A. Diagnosing tuberculosis infection in the 21st century: new tools to tackle an old enemy. *Chest* 2007; 131(6):1898-906.
53. Banks RE. Measurement of cytokines in clinical samples using immunoassays: problems and pitfalls. *Crit Rev Clin Lab Sci* 2000; 37(2):131-82.
54. Marques-Vidal P, Bochud M, Bastardot F, et al. Levels and determinants of inflammatory biomarkers in a Swiss population-based sample (CoLaus study). *PLoS One* 2011; 6(6):e21002.
55. Sindermann J, Kruse A, Frercks HJ, et al. Investigations of the lymphokine system in elderly individuals. *Mech Ageing Dev* 1993; 70(1-2):149-59.
56. Fagiolo U, Cossarizza A, Scala E, et al. Increased cytokine production in mononuclear cells of healthy elderly people. *Eur J Immunol* 1993; 23(9):2375-8.
57. Pahlavani MA, Richardson A. The effect of age on the expression of interleukin-2. *Mech Ageing Dev* 1996; 89(3):125-54.

58. Hager K, Machein U, Krieger S, et al. Interleukin-6 and selected plasma proteins in healthy persons of different ages. *Neurobiol Aging* 1994; 15(6):771-2.
59. Lakatos P, Foldes J, Horvath C, et al. Serum interleukin-6 and bone metabolism in patients with thyroid function disorders. *J Clin Endocrinol Metab* 1997; 82(1):78-81.
60. McKane WR, Khosla S, Peterson JM, et al. Circulating levels of cytokines that modulate bone resorption: effects of age and menopause in women. *J Bone Miner Res* 1994; 9(8):1313-8.
61. Cartier A, Cote M, Lemieux I, et al. Age-related differences in inflammatory markers in men: contribution of visceral adiposity. *Metabolism* 2009; 58(10):1452-8.
62. Fischer CP, Berntsen A, Perstrup LB, et al. Plasma levels of interleukin-6 and C-reactive protein are associated with physical inactivity independent of obesity. *Scand J Med Sci Sports* 2007; 17(5):580-7.
63. Colbert LH, Visser M, Simonsick EM, et al. Physical activity, exercise, and inflammatory markers in older adults: findings from the Health, Aging and Body Composition Study. *J Am Geriatr Soc* 2004; 52(7):1098-104.
64. Petrovsky N, McNair P, Harrison LC. Diurnal rhythms of pro-inflammatory cytokines: regulation by plasma cortisol and therapeutic implications. *Cytokine* 1998; 10(4):307-12.
65. Skogstrand K, Ekelund CK, Thorsen P, et al. Effects of blood sample handling procedures on measurable inflammatory markers in plasma, serum and dried blood spot samples. *J Immunol Methods* 2008; 336(1):78-84.
66. Hosnijeh FS, Krop EJ, Portengen L, et al. Stability and reproducibility of simultaneously detected plasma and serum cytokine levels in asymptomatic subjects. *Biomarkers* 2010; 15(2):140-8.
67. Wong HL, Pfeiffer RM, Fears TR, et al. Reproducibility and correlations of multiplex cytokine levels in asymptomatic persons. *Cancer Epidemiol Biomarkers Prev* 2008; 17(12):3450-6.
68. Friebe A, Volk HD. Stability of tumor necrosis factor alpha, interleukin 6, and interleukin 8 in blood samples of patients with systemic immune activation. *Arch Pathol Lab Med* 2008; 132(11):1802-6.
69. Clendenen TV, Arslan AA, Lokshin AE, et al. Temporal reliability of cytokines and growth factors in EDTA plasma. *BMC Res Notes* 2010; 3:302.
70. Picker SM, Steisel A, Gathof BS. Evaluation of White Blood Cell- and Platelet-Derived Cytokine Accumulation in MIRASOL-PRT-Treated Platelets. *Transfus Med Hemother* 2009; 36(2):114-120.
71. Apelseth TO, Hervig TA, Wentzel-Larsen T, et al. Cytokine accumulation in photochemically treated and gamma-irradiated platelet concentrates during storage. *Transfusion* 2006; 46(5):800-10.
72. Cannon JG, van der Meer JW, Kwiatkowski D, et al. Interleukin-1 beta in human plasma: optimization of blood collection, plasma extraction, and radioimmunoassay methods. *Lymphokine Res* 1988; 7(4):457-67.
73. Panicker G, Meadows KS, Lee DR, et al. Effect of storage temperatures on the stability of cytokines in cervical mucous. *Cytokine* 2007; 37(2):176-9.
74. Jackman RP, Utter GH, Heitman JW, et al. Effects of blood sample age at time of separation on measured cytokine concentrations in human plasma. *Clin Vaccine Immunol* 2011; 18(2):318-26.

75. Leroux-Roels G, Offner F, Philippe J, et al. Influence of blood-collecting systems on concentrations of tumor necrosis factor in serum and plasma. *Clin Chem* 1988; 34(11):2373-4.
76. Aziz N, Nishanian P, Mitsuyasu R, et al. Variables that affect assays for plasma cytokines and soluble activation markers. *Clin Diagn Lab Immunol* 1999; 6(1):89-95.
77. de Jager W, Bourcier K, Rijkers GT, et al. Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays. *BMC Immunol* 2009; 10:52.
78. Butterfield LH, Potter DM, Kirkwood JM. Multiplex serum biomarker assessments: technical and biostatistical issues. *J Transl Med* 2011; 9:173.
79. Flower L, Ahuja RH, Humphries SE, et al. Effects of sample handling on the stability of interleukin 6, tumour necrosis factor-alpha and leptin. *Cytokine* 2000; 12(11):1712-6.
80. Thavasu PW, Longhurst S, Joel SP, et al. Measuring cytokine levels in blood. Importance of anticoagulants, processing, and storage conditions. *J Immunol Methods* 1992; 153(1-2):115-24.
81. Pawliszyn J. Sampling And Sample Preparation For Field And Laboratory *In* Science E, ed. Amsterdam: Elsevier Science, 2002.
82. Bortz J. Forschungsmethoden und Evaluation. 3 ed. Berlin: Springer-Verlag, 2002.
83. Bornefalk E, Ljunghall S, Lindh E, et al. Regulation of interleukin-6 secretion from mononuclear blood cells by extracellular calcium. *J Bone Miner Res* 1997; 12(2):228-33.
84. Hampton MB, Vanags DM, Porn-Ares MI, et al. Involvement of extracellular calcium in phosphatidylserine exposure during apoptosis. *FEBS Lett* 1996; 399(3):277-82.
85. Marie C, Muret J, Fitting C, et al. Reduced ex vivo interleukin-8 production by neutrophils in septic and nonseptic systemic inflammatory response syndrome. *Blood* 1998; 91(9):3439-46.
86. Reinsberg J, Dembinski J, Dorn C, et al. Determination of total interleukin-8 in whole blood after cell lysis. *Clin Chem* 2000; 46(9):1387-94.
87. Tejde A, Mathsson L, Ekdahl KN, et al. Immune complex-stimulated production of interleukin-12 in peripheral blood mononuclear cells is regulated by the complement system. *Clin Exp Immunol* 2004; 137(3):521-8.
88. van Deventer SJ, Buller HR, ten Cate JW, et al. Experimental endotoxemia in humans: analysis of cytokine release and coagulation, fibrinolytic, and complement pathways. *Blood* 1990; 76(12):2520-6.
89. Sikkeland LI, Thorgersen EB, Haug T, et al. Complement activation and cytokine response by BioProtein, a bacterial single cell protein. *Clin Exp Immunol* 2007; 148(1):146-52.
90. Carroll MC. The complement system in regulation of adaptive immunity. *Nat Immunol* 2004; 5(10):981-6.
91. Iba T, Kidokoro A, Fukunaga M, et al. Association between the severity of sepsis and the changes in hemostatic molecular markers and vascular endothelial damage markers. *Shock* 2005; 23(1):25-9.
92. Jensen T, Kierulf P, Sandset PM, et al. Fibrinogen and fibrin induce synthesis of proinflammatory cytokines from isolated peripheral blood mononuclear cells. *Thromb Haemost* 2007; 97(5):822-9.

93. Smiley ST, King JA, Hancock WW. Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4. *J Immunol* 2001; 167(5):2887-94.
94. Liu X, Piela-Smith TH. Fibrin(ogen)-induced expression of ICAM-1 and chemokines in human synovial fibroblasts. *J Immunol* 2000; 165(9):5255-61.
95. Qi J, Goralnick S, Kreutzer DL. Fibrin regulation of interleukin-8 gene expression in human vascular endothelial cells. *Blood* 1997; 90(9):3595-602.
96. Qi J, Kreutzer DL. Fibrin activation of vascular endothelial cells. Induction of IL-8 expression. *J Immunol* 1995; 155(2):867-76.
97. Jennewein C, Tran N, Paulus P, et al. Novel aspects of fibrin(ogen) fragments during inflammation. *Mol Med* 2011; 17(5-6):568-73.
98. Levi M. The coagulant response in sepsis and inflammation. *Hamostaseologie* 2010; 30(1):10-2, 14-6.
99. Paul J, Strickland S, Melchor JP. Fibrin deposition accelerates neurovascular damage and neuroinflammation in mouse models of Alzheimer's disease. *J Exp Med* 2007; 204(8):1999-2008.
100. Seeger FH, Tonn T, Krzossok N, et al. Cell isolation procedures matter: a comparison of different isolation protocols of bone marrow mononuclear cells used for cell therapy in patients with acute myocardial infarction. *Eur Heart J* 2007; 28(6):766-72.
101. Gleissner CA, von Hundelshausen P, Ley K. Platelet chemokines in vascular disease. *Arterioscler Thromb Vasc Biol* 2008; 28(11):1920-7.

7 Abbreviations

EDTA ethylenediaminetetraacetic

EGF epidermal growth factor

ELISA Enzyme-linked immunosorbent assay

ENA-78 Epithelial cell-derived neutrophil-activating peptide-78

FCS fetal calf serum

GCP-2 granulocyte chemotactic protein

IL-8 interleukin-8

IL-1RA interleukin 1 receptor antagonist

GRO-α Growth-related Oncogene alpha

HBBS Hanks balanced salt solution

HI-serum heat inactivated serum

MIP-1α monocyte chemotactic protein-1alpha

MIP-1β macrophage inflammatory protein 1beta

MMP-9 matrix metalloproteinase 9

PBMC peripheral blood mononuclear cell

PDGF platelet-derived growth factor

SEM standard error of the mean

TGF-β transforming growth factor beta

TIMP-1 tissue inhibitor of metalloproteinases

TNF-α tumor necrosis factor-alpha

VEGF vascular endothelial growth factor

MEDIZINISCHE UNIVERSITÄT WIEN

Applied Immunology
Cardio - Thoracic Research in Surgery

Christian Doppler Laboratory
for the Diagnosis & Regeneration of Cardiac and Thoracic Diseases

Stability of cytokines and chemokines in serum and plasma samples: Secretion triggered by serum supplement and fibrin

L. Beer[1,2], M. Mildner[3], K. Hoetzenecker[1,2], G. Werba[1,2], S. Hacker[1,2], S. Nickl[1,2], M. Zimmermann[1,2], A. Mitterbauer[1,2], H. J. Ankersmit[1,2], M. Lichtenauer[1,2]

[1]Department of Thoracic Surgery, Medical University Vienna, Vienna, Austria
[2]Christian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration, Vienna, Austria
[3]Department of Dermatology, Medical University Vienna, Vienna, Austria

Introduction

Chemokines are multifunctional mediators that are involved in development and homeostatic, stem-cell survival, wound healing and immune responses, as well as triggering chemotaxis and angiogenesis. Diagnostic analysis of cytokines and chemokines in serum or plasma has become an important issue in several disease conditions. However, cytokines and chemokines are usually not considered to be very stable after blood collection, which might therefore alter test results. Thus, the aim of the pilot study was to obtain better knowledge about stability of these mediators in blood samples, to investigate factors that might influence chemokines release and further evaluate how chemokines secretion is regulated under these circumstances.

Material and Methods

Venous blood was taken from healthy probands (n=7) using different blood tubes (serum, heparin plasma and EDTA plasma). Blood tubes were either centrifuged initially within 20 minutes after venipuncture and kept frozen at -80° until further testing or were stored at 4°C, at room temperature (RT) or at 37° for up to 24 hours.
Human peripheral blood mononuclear cells (PBMC, n=8) were separated and $1{,}0*10^6$ cells were incubated with increasing levels of autologous serum supplementation (2,5%, 5%, 10% and 20%) or with increasing concentrations of human fibrin (0,25µg, 0.5µg, 10µg, 50µg, 200µg and 500µg). Samples were evaluated for IL-8, IL-6, ENA-78, GCP-2 and IL-1β using commercially available Enzyme-linked immunosorbent assay (ELISA) kits.

Results

A significant difference was detected for GCP-2 in heparin within 4 hours when samples were stored at 4°. All examined mediators rise when samples were stored above room temperature for more than 4 hours in serum tubes. The rise of serum chemokine and cytokine levels culminated in a 335-fold increase for IL-8, a 79-fold increase for IL-6, a 22-fold increase for ENA-78, and a 17-fold increase for GCP-2 compared to basic values. Serum levels of IL-1β were not detectable at basic samples but rise up to 1157 pg/ml (SEM ±415,7 pg/mL).

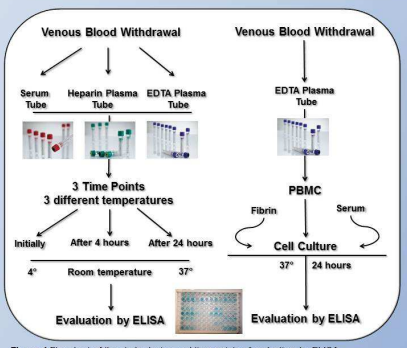

Figure 1 Flowchart of the study design and time points of evaluation by ELISA.
Illustration of blood collection tubes adopted from Greiner Bio One, Austria.

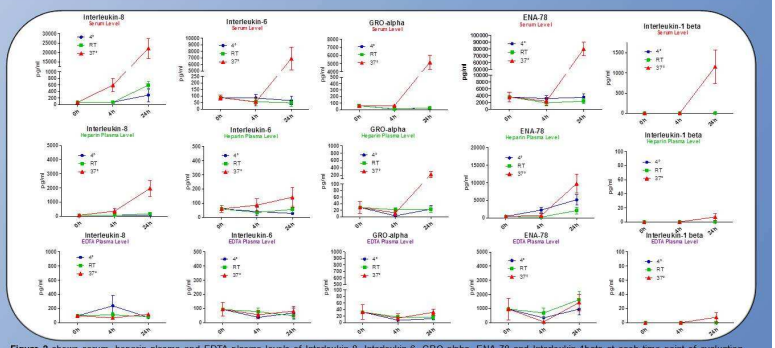

Figure 2 shows serum, heparin plasma and EDTA plasma levels of Interleukin-8, Interleukin-6, GRO-alpha, ENA-78 and Interleukin-1beta at each time point of evaluation (mean ±SEM). Blood tubes kept in a cold environment (4° Celsius) showed stable values for all tested chemokines. Concentration of chemokines evidenced a slight increase in heparin plasma tubes and a strong increase in serum, especially when stored under warm conditions (>room temperature) (n=7).

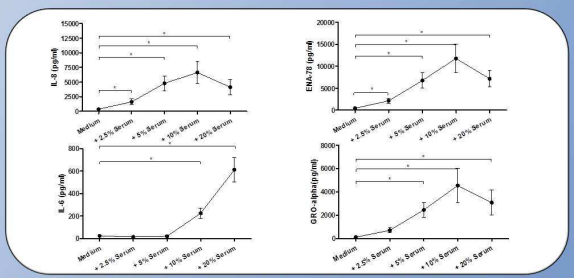

Figure 3: Incubation of peripheral blood mononuclear sells with increasing concentrations of autologous serum induct a massively increase of IL-8, IL-6, ENA-78 and GRO-alpha secretion. This increment was not detectable when cells were incubated in together with 200 µl of autologous heparinized plasma (data not shown) (n=8).

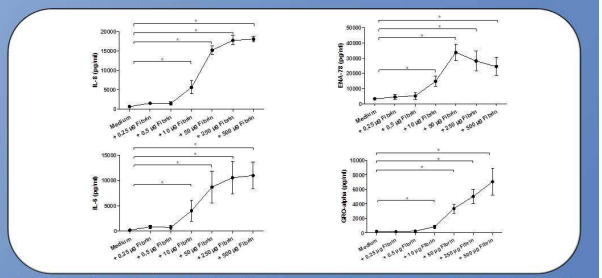

Figure 4: Chemokine secretion triggered by fibrin: When fibrin was added in increasing doses, as significant induction of IL-8, IL-6, ENA,-78 and GRO-alpha was detected. A highly significant induction of these mediators was measured when 50 µg or more of fibrin were added to $1{,}0*10^6$ cells per ml. Maximal induction of cytokine release seems to be at 250 to 500 µg of fibrin, whereas higher levels do not further stimulate cytokine secretion (n=8).

Discussion

These data indicate that chemokine levels remain stable when analysed within a short interval after venipuncture or when tubes are stored at 4°. Chemokine levels in EDTA plasma emerged to be most stable, which seems to be related to the fact that calcium is an important co-factor for cytokine release. Whereas cytokine levels increased significantly in serum tubes exposed to temperatures higher than 24° (RT). We indicated that accumulation of elevated chemokines levels in serum samples are partly regulated by coagulation products such as fibrin.

Curriculum Vitae

Lucian Beer

PERSONAL BACKGROUND

Nationality: Austrian
Family Status: Single
Date and Place of Birth: May 16th, 1989, Vienna, Austria

EDUCATION

1995 – 1999	Primary School
1999 – 2007	Erzbischöfliches Real und Aufbaugymnasium Hollabrunn, Austria (High School)
2007/06	Matura (high school graduation) with Distinction
2007 – 2008	Zivildienst
2008/10 – Present	Medical Student at the Medical University of Vienna, Austria
2009/11 – Present	Student Research Fellow at the Department of Cardio-Thoracic Surgery, General Hospital Vienna, Medical University of Vienna, Austria

---

CLINICAL TRAINING

2010/09	Clinical Clerkship at the Department of Internal Medicine, LKH Hollabrunn, Austria (4 weeks)
2010/08	Clinical Clerkship at the Department of Surgery, LKH Hollabrunn, Austria (2 weeks)
2011/02	Clinical Clerkship at Surgery of Dr. Michael Putz; Göstling an der Ybbs, Austria (2 weeks)
2011/08	Clinical Clerkship at the Department of Gynaecology, LKH Tulln (2 weeks)
2011/09	Clinical Clerkship at the Department of Paediatrics, LKH Tulln (2 weeks)
2011/09	Clinical Clerkship at the Department of Ambulance Station; LKH Korneuburg (2 weeks)
2012/02	Clinical Clerkship at the Department of Cardiac Surgery; AKH Vienna (2 weeks)

CONTINUING EDUCATION

2011/11	Methodenseminar “Statistik” – Methods Seminar “Statistics” MMag. Dr. Alexandra Graf
2012/02	Biometrie I: Beschreibung und Visualisierung medizinischer Daten – Biometry I: Description and Visualization of Medical Data, Vienna, Austria
2012/02	Biometrie II: Statistische Tests und Lebensdaueranalyse bei medizinischen Fragestellungen – Biometrie II: Statistical Tests and Analysis of Survival in Medical Research, Vienna, Austria

2012/05	Methodenseminar: “Fluoreszenzbasierte Methoden in der zellbiologischen Forschung“– Methods Seminar „Fluoreszenz Based Methods in cell-biological science“

RESEARCH ACTIVITY AND PUBLICATIONS

Lichtenauer M, Mildner M, Baumgartner A, Hasun M, Werba G, Beer L, Altmann P, Roth G, Gyöngyösi M, Podesser BK, Ankersmit HJ.: **Intravenous and intramyocardial injection of apoptotic white blood cell suspensions prevents ventricular remodelling by increasing elastin expression in cardiac scar tissue after myocardial infarction**. Basic Res Cardiol. 2011 Mar 17

ABSCTRACTS AND POSTER PRESENTATIONS

L. Beer, K. Hoetzenecker, M. Hasun, A. Baumgartner, S. Hacker, M. Wolfsberger, A. Mangold, S. Nickl, M. Zimmerman, A. Mitterbauer, B. K. Podesser, H. J. Ankersmit, M. Lichtenauer.: **Serum-free Cell Culture Medium Reduces Myocardial Damage After Ischemia in an Experimental Model of Myocardial Infarction: Importance for Cell Therapeutic Methods.** Austrian Journal of Cardiology 2010; 17 (5-6), 165-233.

K. Hoetzenecker, S. Hacker, A. Mitterbauer, L. Beer, M. Rauch, W. Hotzenecker, E. Guenova, M. Lichtenauer, W. Kleptko, H. K. Ankersmit,: **Expansion of a unique, lung-specific autoreactive T helpe cell polpulation in COPD**. European Surgery, Acta Chirurgica Austriaca, Volume 42, Suppl 236, 2010.

K. Hotzenecker, M. Töpker, M. Rauch, L. Beer, S. Hacker, M. Zimmermann, W. Klepetko, H. J. Ankersmit.: **Seldom referral to the thoracic surgeon: spontanously ruptured left inferior thyroid artery**. European Surgery, Acta Chirurgica Austriaca, Volume 42, Suppl 236, 2010.

L. Beer, G. Werba, S. Nickl, M. Zimmerman, A. Mitterbauer, H. J. Ankersmit, M. Lichtenauer: **Secretion of cytokines and chemokines by peripheral blood mononuclear cells is triggered by coagulation products**. ÖKG 2011, Salzburg – Congress, *Salzburg*

L. Beer, G. Werba, S. Nickl, M. Zimmerman, A. Mitterbauer, H. J. Ankersmit, M. Lichtenauer: **Secretion of cytokines and chemokines by peripheral blood mononuclear cells is triggered by coagulation products**. ÖGIM 2011, Wien Klein Wochenschr (2011) 123/17-18;

G. Werba, M. Mildner, A. Baumgartner, L. Beer, M. Gyöngyösi, B. K. Podesser, H. J. Ankersmit, M. Lichtenauer.: **Anti-thymocyte globulin (ATG) reduces damage caused by ischemia and preserves cardiac function after experimental myocardial infarction**. European Surgery, Acta Chirurgica Austriaca; Volume 43, Suppl 242/11, 2011.

L. Beer, G. Werba, S. Nickl, M. Zimmerman, A. Mitterbauer, H. J. Ankersmit, M. Lichtenauer: **Secretion of cytokines and chemokines by peripheral blood mononuclear cells is triggered by coagulation products**. European Surgery, Acta Chirurgica Austriaca; Volume 43, Suppl 242/11, 2011.

Michael Lichtenauer, Lucian Beer[1], Michael Mildner, Matthias Zimmermann, Bruno Karl Podesser, Wolfgang Sipos, Erwin Tschachler, Mariann Gyöngyösi, Hendrik Jan Ankersmit: **Secretome of apoptotic peripheral blood cells (APOSEC) confers cytoprotection to cardiomyocytes and inhibits tissue remodelling after acute myocardial infarction.** European Surgery, Acta Chirurgica Austriaca; Volume 43, Suppl 243/11, 2011
[1]presenting author

L. Beer, T. Szerafin, A. Mitterbauer, T. Haider, G.A. Roth, M. Dworschak, H. J. Ankersmit.: **Continued lung ventilation during open heart surgery attenuates systemic heat-shock protein 70 release.** European Surgery, Acta Chirurgica Austriaca. 2012, *in press*

L. Beer, T. Szerafin, A. Mitterbauer, T. Haider, G.A. Roth, M. Dworschak, H. J. Ankersmit.: **Continued ventilation during open heart surgery reduces systemic secretion of soluble ST2.** European Surgery, Acta Chirurgica Austriaca. 2012, *in press*

A. Mitterbauer, T. Szerafin, L. Beer, T. Haider, G.A. Roth, M. Dworschak, H. J. Ankersmit.: **Can continued ventilation during open heart surgery inhibit polymorphonuclear cell activation?** European Surgery, Acta Chirurgica Austriaca. 2012, *in press*

L. Beer, G. Werba, S. Nickl, M. Zimmerman, A. Mitterbauer, H. J. Ankersmit, M. Lichtenauer: **Stability of cytokines and chemokines in serum and plasma samples**. European Surgery, Acta Chirurgica Austriaca. 2012, *in press*

CONGRESSES AND MEETINGS

2010/12	2nd EACTS Meeting on Cardiac and Pulmonary Regeneration, *Vienna*
2011/5	Jahrestagung der Österreichischen Kardiologischen Gesellschaft; *Salzburg*
2011/10	Jahrestagung der Österreichischen Gesellschaft für Innere Medizin; *Innsbruck*
2011/10	Jahrestagung der Österreichischen Gesellschaft für Transplantation, Transfusion und Genetik; *Graz*
2011/10	35. Seminar der Österreichischen Gesellschaft für Chirurgische Forschung, *Wagrain*; Salzburg
2012/5	Jahrestagung der Österreichischen Kardiologischen Gesellschaft; *Salzburg*
2012/6	53. Kongress Österreichische Gesellschaft für Chirurgie, *Salzburg*

AWARDS AND GRANTS

2009/12	Leistungsstipendium – Medical University Vienna
2010/12	Leistungsstipendium – Medical University Vienna
2011/12	Leistungsstipendium – Medical University Vienna
2007/06	Matura (High School Graduation) with Distinction

TEACHING ACTIVITY

2011/12 – Present Teaching Assistant at the Department of Physiology, General Hospital Vienna, Medical University of Vienna, Univ. Prof. Ivo Volf

---

MEMBERSHIPS

2011/12 Austrian Society of Transplantation, Transfusion and Genetics

---

EXCURSIONS

2011/4/28-29 Kaposvar; APOSEC study

CURRENT STUDIES

Diploma thesis on triggers of chemokine release of peripher blood mononuclear cells
Collagen induces Arthritis – Mice model
Mechanical lung ventilation during cardiopulmonary bypass – immunological effects

---

METHODS

Cultivation of human cell lines
ELISA
Flow Cytometry