Master Thesis

Feasibility study for different sample procedures Illustrated with Lipocalin-2

Submitted by

Ilona Brandstätter

Fort the academic degree of

Master of Science in Biobanking

(MSc)

at the

Medical University of Graz

Executed at

Biobank Graz

Under the supervision of

Univ.-Prof. Dr. Berthold Huppertz

Submitted 2018

Graz, 2018/7/3

Statutory Declaration

I declare on my honor that I have written this dissertation independently and without assistance, that no source other than those cited were used and that the sources used verbatim or in substance have been marked as such.

Graz, 2018/7/3 eh Brandstätter Ilona

Acknowledgement

Writing this master theses has had a big impact on me. It was a period of intense learning, not only in the scientific area, but also on a personal level.

I would like to thank Univ.-Prof. Dr. Berthold Huppertz for his patience and assistance as my supervisor and Univ.-Prof. Dr. Barbara Obermayer-Pietsch for providing all resources and for financing this thesis.

Special thanks go to my family. My parents, who brought me up to believe that I can achieve everything if I am willing to work for it. My husband Bernhard and my son Jan, who supported me during my studies and helped me whenever they could.

Zusammenfassung

Für die Planung von klinischen Studien ist es notwendig unterschiedlichste Probenbearbeitungsprozesse zu testen um die Stabilität von Biomarkern in Serum und Harn zu untersuchen. Hierzu wurde das Neutrophile Gelatinase-Associated Lipocalin (NGAL) als etablierter Biomarker vermessen. NGAL ist ein Lipoprotein das in verschiedensten Geweben im Körper produziert wird und vor allem als Routineparameter bei akutem Nierenversagen gebräuchlich ist. Da die Probengewinnung und zeitnahe Verarbeitung der Proben für Studien, bei sich in Intensivpflege befindlichen PatientInnen, sehr schwierig ist, war es notwendig potenziell vorkommende Einschränkungen in der Probenbearbeitung vorab auf ihre Auswirkungen auf die NGAL Konzentration in Serumproben und Harnproben zu testen, um eine ausreichend gute Probenqualität sicherzustellen.

Serumproben und Harnproben von neun PatientInnen wurden gesammelt und in fünf Gruppen aufgeteilt. Gruppe A, als Goldstandard konzipiert, folgte allen geltenden Richtlinien für GLP (Good Laboratory Practice), wurde also in kürzest möglicher Zeit im Labor verarbeitet, aliquotiert und bei -80°C tiefgefroren. Gruppe B verblieb nach der Zentrifugation noch für 48 Stunden im Kühlschrank und wurde danach aliquotiert und bei -80°C eingefroren. Das, in der Praxis leider vorkommende, wiederholte Auftauen und Einfrieren während der Lagerung wurde mit Gruppe C getestet. Die Gruppen A, B und C wurden nach sechs Monaten vermessen, während die Gruppen E und D, die gleich verarbeitet wurden wie Gruppe A, jeweils nach elf Monaten gemessen wurden. Gruppe E war bei -80°C eingefroren während Gruppe D bei -20°C eingefroren wurde. Innerhalb dieser Studie wurden auch zwei unterschiedliche ELISA Kits getestet und verglichen.

Die Präzision der Kits wurde evaluiert und mit den Herstellerangaben verglichen. Beide ELISAs zeigten gute Messgenauigkeit und Reproduzierbarkeit der Ergebnisse. Die Evaluierung des Einflusses von verzögertem Einfrieren oder mehrmaligem Auftauen und Einfrieren auf die Probenqualität zeigte keine signifikanten Unterschiede. Die Testergebnisse bei unterschiedlichen Lagertemperaturen und einer Lagerungsdauer von sechs beziehungsweise elf Monate waren fragwürdig und sollten noch weiter analysiert werden. Speziell die gemessene

Konzentrationszunahme bei Gruppe E war, wenn auch nicht signifikant, überraschend groß.

Während in dieser Studie NGAL als Biomarker eine hervorragende Stabilität gegenüber einer kurzen Lagerung bei nur 4°C und mehrmaligem Auftauen und Einfrieren zeigt, so scheint die Lagerung über einen längeren Zeitraum doch messbaren Einfluss zu haben. Gründe für die Konzentrationszunahme bei Lagerung für elf Monaten konnten keine gefunden werden. Sollten die Messungen für eine Studie länger als sechs Monate nach Probensammlung geplant sein sollte vorher eine nochmalige Austestung von Proben stattfinden.

Abstract

For planning clinical studies it is important to investigate different sample handling procedures to determine the stability of markers in serum and urine samples. Neutrophil Gelatinase-Associated Lipocalin (NGAL) was used as an established biomarker. NGAL is a lipoprotein that is expressed in various body tissues and is mostly known as a biomarker for AKI, acute kidney injury. Since sample collection and handling is rather difficult with patients being under intensive care, this feasibility study tested different possible restrictions for sample handling procedures and their effects to guarantee sample quality.

From nine patients serum and urine samples were collected and divided into five groups. Group A, used as the golden standard, was following all recommendations of GLP (Good Laboratory Practice) guidelines. The samples were transported to the laboratory, processed and aliquoted as fast as possible and immediately frozen at -80°C. Group B was stored at 4°C for 48 hours before aliquoting and freezing at -80°C. Group C was processed like group A but was thawed and refrozen twice during storage. Groups A, B and C were analyzed after six months while groups D and E were handled as group A while group D was stored at -20°C and group E at -80°C for eleven months. Two different ELISA assays were tested and compared in this feasibility study.

Assay performance was evaluated and compared with provided information from the manufacturers. Both assay showed good performance and reproducible results. The evaluation of measurable effects of delayed freezing or two thawing and freezing cycles during storage on sample quality showed no significant difference. Measured results after storage with different temperatures and duration of six respectively eleven months seem to be questionable and might need further testing. Especially results of group E which show an increase of concentration were, if not significant, rather surprising.

While in this study NGAL shows remarkably stability after short storage at 4°C and multiple thawing and freezing cycles, long term storage seems to effect the concentration measurably. Since no reasons could be found for the increase in

concentration after storage for eleven months it is advisable to rerun testing if study sample analysis would start after more than six months of storage.

Table of contents

Abbreviations

AKI	Acute Kidney Injury
ANOVA	Analysis of Variance
BRISQ	Biospecimen Reporting for Improved Study Quality
CEN	European Committee for Standardization
CENELEC	European Committee for Electrotechnical Standardization
CV	Coefficient of Variation
EFTA	European Free Trade Association
ELISA	Enzyme-Linked Immuno Sorbent Assay
ETSI	European Telecommunications Standards Institute
FFPE	Formalin-Fixed Paraffin-Embedded
GCP	Good Clinical Practice
GCLP	Good Clinical Laboratory Practice
GLP	Good Laboratory Practice
IC	Informed Consent
ICH	International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use
ISBER	International Society of Biological and Environmental Repositories
ISO	International Organization for Standardization
KKS	Koordinierungszentrum für Klinische Studien (Coordination Center for Clinical Studies)
NGAL	Neutrophil Gelatinase-Associated Lipocalin
NIH	National Institutes of Health
OECD	Organization for Economic Co-Operation and Development
SCI	Science Citation Index
SOP	Standard Operation Procedure
TS	Technical Standards

List of figures

List of tables

1. Introduction

Medical innovation is based on well designed and performed research studies and clinical trials to provide reliable results. In the last decades laws and regulations were legislated to protect patients and researchers and enable an environment where medical research is taking place under analytically and ethically correct conditions. Especially ethics and data protection became very important in the last years, while sample handling and correctness of analytical data calculation and statistics still seem to be less important. While protection of patients' rights and ethical considerations are clearly important, it is also important to reliably produce research results and data. Over the last five years the numbers of retracted publications are increasing because of irreproducible results, wrong statistical calculations and inappropriate scientific behavior [1]. Hence, there is a need not only to increase awareness for ethically approved studies and data protection, but also to enable research based on high quality samples and data.

National and international laws, regulations and standards are being developed, but still the quality of research publications seems to decrease rather than improve. What are the possible reasons for this development and how can it be stopped?

Statistical errors and wrong data interpretation are mostly due to lack of respective training, knowledge and time. Low sample quality, which is believed to be another important type of failure, is a product of different errors and misbeliefs. In most routine laboratories SOP's (Standard Operation Procedures) are used to guarantee high sample quality and correct short term storage. However, long time storage does not seem to be so important, as there are mostly no clear standards for this. Also, there are no definite standards for research samples, their handling and their storage.

Sample analysis as the core responsibility in routine laboratories follows strict regulations, concerning timelines and data protection and always is patient and result orientated. Anonymization or aliquoting samples for any other reason but following contemporary analysis are usually not part of routine standard procedures. The advantages of implementing research in routine laboratories are the most up-to-date equipment, infrastructure and trained technicians, who rarely need further training. The disadvantages are that samples are collected from routine rests very often without any information for the routine staff about special conditions or planned tests, hence the samples are stored in an available freezer without aliquoting and sometimes even non-labeled. Implementing research in routine infrastructure is clearly useful but there needs to be a strict separation in procedures, storage and a better communication between routine staff and researchers.

To overcome all these difficulties biobanks and specialized repositories are funded all over the world. Specialists in different fields work together for the best results, sharing experience and knowledge. The scope is to guarantee high quality of samples with reliable data recording concerning each aspect of sample collection, handling and storage. Additionally, medical history and even data about lifestyle can be collected and provided. Research is not implemented in routine testing but becomes routine by providing standard procedures, guidelines, and information concerning each aspect from study design to statistical calculation.

1.1 Neutrophil gelatinase-associated lipocalin

In this study, neutrophil gelatinase-associated lipocalin (NGAL), was used as a protein marker in blood that is already in clinical use for certain clinical questions but is currently investigated to be established as a biomarker for a higher number of differential diagnoses.

NGAL, also known as Lipocalin-2, is a small size protein expressed by neutrophils and in different organs like kidney, prostate and epithelia of respiratory and alimentary tracks in low levels. NGAL is member of a family of proteins that bind and transport small hydrophobic ligands [2].

Today, NGAL is mostly known as a biomarker for acute kidney disease (AKI) since in comparison to serum creatinine, which is also used as a parameter for kidney functionality, the concentration of NGAL increases after only two hours after injury in blood and urine. Additionally, the levels of NGAL are associated with the severity of organ trauma and therefore the prognosis is directly related to NGAL as well [3].

Recently, NGAL has been investigated and discussed as a biomarker for inflammation and cancer. NGAL binds to bacterial siderophores and initiates immune responses to the infection while at the same time inhibits bacterial growth by sequestering iron-laden siderophores. The regulation of NGAL expression is still mostly unknown but ranges from pro-inflammatory cytokine, tumor necrosis factor alpha to interferons [4].

NGAL also seems to be important in the iron metabolism and studies are running now on the role of NGAL in cardiovascular disease and the possibilities of new therapies for iron deficiency for hemodialysis patients and therapeutic iron depletion for iron overload [5, 6, 7].

1.2 Good Clinical Laboratory Practice (GCLP)

Good Clinical Laboratory Practice (GCLP) guidelines are merging standards from GLP (Good Laboratory Practice) and GCP (Good Clinical Practice) and were developed as an approach of utilizing already existing standards.

GCP is an international ethical and scientific standard to ensure the rights and well-being of trial participants that has its origin in the Declaration of Helsinki, ICH (International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use) GCP Guideline. GLP was developed to ensure high quality of test data and covers the organizational aspects of laboratory performance, monitoring, recording and reports (OECD GLP Guideline) [8].

The aim of the development of these new standards was to have a single document available that includes national and international regulatory authorities' guidance as well as directives of other organizations to have a set of standards for laboratory testing in human clinical trials. These standards include operational disciplines, quality management, sample management and data management.

The framework of GCLP also includes specific regulations for materials, samples, reagents and equipment used in laboratories analyzing data for clinical trials.

In general, samples should be fit for the study purpose. The collection and processing needs should follow SOPs developed for the study and approved by the study management.

Analytical methods are to be described, tested and the performance recorded. Data about staff qualification, quality controls and equipment safety has to be collected and reported to the study management [9].

For sample quality this includes for example that the use of temperature logs is necessary, especially during shipment if the samples are not collected on site, and during storage until measurement is finished. Only with valid temperature control can it be guaranteed that the samples were kept at the mandatory temperature to ensure stable sample quality. Samples should be labelled, preferably with barcodes, printed or craved on the tubes to ensure distinctiveness and label durability. Poorly labelled samples or samples with missing labels should not be analyzed until confirmation of the study management about correctness is available. Study design has to contain what test kits are used to clarify whether the appropriate criteria are fulfilled. Documentation should include all study results, quality management data e.g. sell-by date, standard curves or control results. Additionally, recorded data should include the date and time of analyzes and the analyzing person so each individual result is verifiable. Any changes or corrections made during the study have to be reported and recorded to allow a follow up. Data should be stored as raw data and study result, both following legal data protection regulations. After the study is finished the samples have to be destroyed for ethical reasons except the informed consent (IC) signed by all study participants specifically includes storage and further use after the study [10].

All these regulations are strongly recommended by different international organizations who are involved in public health or other research fields but there is no control from any authority specifically named. Guidelines are often formulated as ‘Shoulds’ and ‘Help’ with no consequences if not followed. Also audits by external parties are recommended but not stipulated unless for an ISO certification (International Organization for Standardization).

Although they are general recommendations, these guidelines seem to rather focus on large research facilities with specialized staff and defined study infrastructure rather than small scale studies performed in a routine facility. Storage for example can become an unexpected risk for sample quality if freezers that are not locked can be opened by any staff member. Samples might be moved in and out and the temperature of the freezer may only be controlled in routine once a day or even less frequent.

Depending on study design there are two main strategies to collect study samples. Participants, who are selected beforehand by specified terms and needs (e.g. disease, health-status, age) are called in to collect the needed samples and documents. This way the sample collection is easier to plan and organize, the samples are processed by trained staff following SOPs and guidelines and therefore are usually of good quality concerning their handling, processing and storage.

Samples which are collected during routine health checks or examinations undergo routine processing first because the patient welfare always is of higher priority than research. After routine analyses the rest of the collected samples can be used for research, if there is a valid informed consent. These research samples are usually stored at room temperature for direct measurements or in a fridge (4°C) until the measurements are finished and the results are released. A second possibility is to take an additional blood sample from the patient during routine blood drawing to enable processing of a research sample immediately. Sample quality is questionable after hours of unknown storage conditions and also these samples are often frozen without aliquoting and thus samples are thawed and frozen several times. Depending on study questions and parameter stability that might be of no concern but it is still no good laboratory practice. Missing aliquoting also leads to significant sample quality concerns if during the study analyses not all parameters can be measured in one run. Samples are thawed and refrozen depending on how many runs of different analyses are needed. Again, this might not lead to significant consequences but should be avoided to ensure good sample quality.

1.3 Replicability crisis

In science reliable knowledge is the foundation of new developments and innovations. In the last century research in the biomedical field has led to an enormous amount of discoveries and promising findings. However, in the last ten years a problem was recognized that threatens to damage the current system: The increasing number of retractions of scientific publications due to questionable results in basic research.

Monya Baker and coauthors did a survey with over 1,500 scientists about reproducibility of experimental results from their own group and from other scientists. They discovered that 70% of the scientists failed to reproduce results and 30% even failed to reproduce their own experimental data. Still over 73% believe in their field of science while only half of the publications seem to be trustworthy [11].

The retraction of scientific publications from the Science Citation Index (SCI) was analyzed in 2014. It shows an increase from 2.0% in the year 2004 to 17.5% in the year 2014. Most papers (18%) are retracted in the first year after their publication and over 50% after two years. The reasons were stated to be basic research failures and unwanted mistakes [12].

The reasons for this ongoing crisis are clearly divided in two sections, one being the system of research and publication and the other the poor training and study management in the scientific field.

Begley and Ioannidis stated in their review that one of the main reasons for the crisis is " *A system that is willing to overlook and ignore lack of scientific rigor and instead rewards flashy results that generate scientific buzz or excitement"* (Begley and Ioannidis,2015) [13].

The pressure to publicize and to produce significant results leads to methods like non-blinded research or running different experiments and choose the one with the best results, which is called cherry picking or p-hacking. Scientists also blame poor study management and insufficient oversight and mentoring for the increase in unreliable results. Not enough statistical power or poor laboratory performance are also named by about 50% of researchers to have led to problems in reproducibility [14].

Especially in the field of biomarkers recently the quality of specimen came into focus to be at least part of the crisis. A majority of researchers is complaining about finding enough specimens of sufficient quality and therefore is questioning their own published data. In most publications data about fundamental methodology concerning specimens is not published, which makes it impossible to reproduce the results [15].

Following this controversy it still needs to be taken into account that basic research still is the foundation for medical innovations and discoveries. As long as raising money for studies is easier when the scientist has a long list of publications and stakeholders only aim for break through results this crisis will continue.

1.3.1 Ways out of the crisis

To improve scientific practice on a grand scale it has been discussed that institutional organizations and the need for their leadership have to be enforced. The institutions could take the responsibility for scientific staff training, providing specialists for study design and management. They can also take the lead in validation study results with investigation, audits and individual laboratory controls at important time points of the studies. Another great advantage of institutional organizations would be possible data sharing, cross-validations and interdisciplinary co-working [13].

Some researchers do not agree with reviewing methods and standardization because running programs might be affected. They also might believe that self-monitoring is sufficient but the facts cannot be ignored and something needs to be changed. Successful research starts with accurate planning every step from sample selection to correct result analysis. When using specimens in medical research laboratory specialists need to be involved in study design to make sure that the quality of samples and chosen analysis methods fulfill necessary quality aspects. While the selection of participants stands at the very beginning, correct sample processing, storage and method selection ensures the quality of samples and study results. Communication between specialists and willingness to divide responsibilities is necessary to produce reliable study results.

Additionally, some organizations try to establish new standards and guidelines to provide help in study design, sample handling and statistics. For example the Biospecimen Reporting for Improved Study Quality (BRISQ) offers guidance in study design, data analysis and specimen handling. Based on three Tier formats they divide specimen data in most important (Tier 1), items advisable (Tier 2) and additional items (Tier 3). For example: Tier 1 would be the way the specimens were stabilized and preserved, Tier 2 would be demographic annotation and Tier 3 would be the type of storage container. BRISQ can also provide SOPs for standard procedures like sample collection or storage conditions [16].

Improving the education of young researchers in experimental methods and scientific writing is what the National Institute of Health (NIH) is doing to address the crisis. They fund online training modules for experimental design, statistics and also enforce ethics discussions among the students [14].

In Austria the institutional organization for improve scientific quality is the Coordination Center for Clinical Studies (KKS / Koordinierungszentrum für Klinische Studien). Their goals are to improve research infrastructure, research staff education and training and to increase public funding. They focus also on participant recruiting improving the public image of basic research [17].

1.4 Biobanking

To overcome small scale sample storing and doubtful quality of research samples, biobanks were founded all over the world. Different scopes and aims led to specialization but all biobanks and repositories follow one goal, to collect and store high quality samples and data. Depending on owner policy samples and data are provided for researchers internally, nationally or internationally. Also virtual biobanks are becoming more important nowadays, not providing samples but assisting investigators in locating special samples and data for their studies from different biobanks all over the world by using specialized software or web portals. Based on networks and cooperation like the International Society of Biological and

Environmental Repositories (ISBER) over the last years biobanks also started to implement working standards. The scope is to collect, store and provide samples and data of high and comparable quality for researchers, scientists and companies [18].

While ISO standards are internationally acknowledged, CEN (European Committee for Standardization) standards are valid only in Europe. Together with CENELEC (European Committee for Electro technical Standardization) and ETSI (European Telecommunications Standards Institute) CEN is officially recognized by the European Unit and the EFTA (European Free Trade Association) to develop and define European standards. One of the sectors CEN is responsible for is healthcare and therefore includes standards for biobanking.

The standards CEN provides are covering different fields in biobanking like products, material, services and processes. Members are encouraged to share knowledge, experiences and strategies for good practice. Very specific standard procedures are called TS (Technical Standards). Today, there are nine different CEN/TS for biobanking, each of them specific for a human material or method (e.g. FFPE tissue, venous blood or metabolomics) or one specific component like isolated DNA or isolated proteins [19].

Networks and cooperation between biobanks are set up to ensure high and comparable quality. However, even with these improvements there is still a long way to go. Regulations and standards are not mandatory and not all biobanks and repositories have yet implemented the respective working processes and standards.

Why critics might disapprove of paying for samples biobanks follow the same ethical rules as any other researcher. Patients sign an informed consent applying to all valid laws and regulations but with no restriction to a specific study. Thus, the samples can be used for any study in the future. Researchers and companies have to present valid ethical commission to receive samples with or without related data. Biobanks also provide services for individual sample collections such as storage, study design and data analysis.

As an example, Biobank Graz is a non-profit organization that only charges the expenses (e.g. laboratory staff, equipment or disposables) but does not charge the sample itself. Hence, especially for studies on rare diseases or specific lifestyle questions biobanks can provide high quality samples and related data [20].

1.5 Feasibility studies

Feasibility studies are smaller scale studies over a short time period and with a lower budget. The role of pilot studies is to examine different facets like recruitment, randomization, processes or implementation of interventions, of a planned larger scale study. Feasibility studies or pilot studies are not preliminary tests but designed to test feasibility, consistency and reliability.

At this point study design and protocols can be tested and if they are deemed infeasible, they can be modified and changed or even removed without any greater consequences. All participating parties can benefit by strengthening their competencies, training their skills and practice, also data can be investigated for integrity. If different institutions or companies are part of a study it is also valuable to test sample transportation or data transmission safety.

Pilot studies need no calculated sample size and therefore can be limited to the necessary size for process feasibility examination hence budget constraints can also be taken into consideration. Data generated in a pilot study should never be included in the larger scale study due to protocol changes and possible unknown variables.

Inferential statistics to provide p-values are not part of a pilot study design because of the small sample size and the lack of hypothesis testing [21].

Lancaster et.al (2004) " *A well-conducted pilot study, giving clear list of aims and objectives within a formal framework will encourage methodological rigor, ensure that the work is scientifically valid and publishable, and will lead to higher quality*." [22]

1.6 Project description and hypothesis

For a planned study in the University Hospital of Graz different sample collection and storage conditions were investigated. This pilot study was performed to test the feasibility of a planned clinical study and the reliability of the collected samples concerning possible pre-analytical variables. Also the performance and the usability of two possible assays were tested. The conclusions are a recommendation for one of the tested assays and what conditions, handling procedures and storage temperatures are acceptable to guarantee a successful study outcome.

The central questions of the feasibility study are:

If and how different handling and storage procedures are effecting the sample quality regarding the NGAL concentration in serum and urine samples?

Which one of the two available ELISA assays shows better performance, higher reliability and is easier to use for this study?

2. Material and Methods

2.1 Sample collection

The serum and urine samples were collected in the ambulance of the Department of Endocrinology at the LKH University Hospital Graz, Austria. The serum and urine samples were part of routine controls and only sample rest volumes were used for this study. Nine patients were selected randomly and from each patient samples from one serum tube and one urine tube were collected. All samples were anonymized and transported to the routine laboratory for endocrinology in maximally 15 minutes. Following the standard laboratory procedure all samples, serum and urine, were numbered, centrifuged at 4000rpm for ten minutes at 10°C, aliquoted in storage tubes of 300µl each and processed as described below. Different time points for freezing, storage temperatures or possible thawing were defined, all of which might occur during a clinical study. In the study groups where samples were immediately frozen, it took maximally 30 minutes between arrival of the samples in the laboratory and storage in freezers at -80°C. In the group where samples were kept cold prior to freezing, tubes were kept cool in a laboratory fridge at 4°C for 48 hours before freezing. In the group were thawing of samples was performed during storage, thawing cycles took 3 hours at room temperature, allowing slow thawing and standing at room temperature before freezing the samples again. The aliquots were numbered and marked with letters (A, B, C, D, E) and S (Serum) or H (Harn = Urine). The letters marked the groups according to the different processing strategies (see 2.1.1).

2.1.1 Study groups:

A: standard laboratory procedure, aliquoting and immediate storage at -80°C for six months

B: standard laboratory procedure, aliquoting, storage at 4°C for 48h and then storage at -80°C for six months

C: standard laboratory procedure, aliquoting and storing at -80°C; after 5 weeks the first thaw and freeze cycle (3 hours at room temperature) and after 6 weeks a second thaw and freeze cycle (3 hours at room temperature), before being stored at -80°C again; total storage time of six months

D: standard laboratory procedure, aliquoting and immediate storing at -20°C for 11 months

E: standard laboratory procedure, aliquoting and immediate storing at -80°C for 11 months

```
Collection                              1. Analysis                              2. Analysis
A  I------------------------------------------I    Storage -80°C for 6 months
B  I 48h -4°C -------------------------- --I    Storage -80°C for 6 months
C  I---thaw/freeze---thaw/freeze------I    Storage -80°C for 6 months
D  I-------------------------------------------------------------------------------------I Storage -20°C for 11 months
E  I-------------------------------------------------------------------------------------I Storage -80°C for 11 months
```

Figure 1: Timeline of the collection and storage procedures of the study groups

2.2 Assays

Two different ELISA kits were used from two different companies. The first assay is the Quantikine ELISA Human Lipocalin/NGAL Immunoassay manufactured by R&D Systems, Inc. The second assay is the Human NGAL ELISA kit manufactured by BioPorto Diagnostics. Both assays are for research purposes only and are not to be used for diagnostic procedures. The assays will be described in detail in 2.3.1 and 2.3.2, the recommendations for sample collection and processing, test performance including precision, specificity and sensitivity are quoted from the instruction sheets of the manufacturers.

The assays were carried out manually except the washing steps, which were done by the ELx50. The reading at 450nm was carried out by the EL808 using GEN 5 Data Analysis Software.

2.2.1 ELISA 1 Human NGAL ELISA

The Human NGAL Testkit by BioPorto is a 3 hour sandwich ELISA, able to detect NGAL in serum and urine samples, performed in a microplate precoated with a monoclonal antibody to human NGAL. Free NGAL present in the samples will bind to the coated wells while unbound materials are washed out. The bound NGAL is then labeled with biotinylated monoclonal detection antibody, unbound detection antibodies are washed out. In a conjugate solution HRP-conjugated streptavidin is added to each well and allowed to form a complex with the biotinylated antibody. Again all unbound materials are cleared out by a washing step. After adding a color-forming peroxidase substrate the bound HRP-streptavidin reacts with the substrate and generates a color product. The color intensity is measured with an ELISA reader at 450nm. All other supplies required are standard laboratory equipment. The following sample collection and storage conditions are recommended by the company. For the samples the end results are given in pg/ml.

2.2.1.1 ELISA 1 sample recommendations

(Quoted from the instruction sheet of the manufacturer [23])

Serum:

Collection in standard serum tubes and processing after standard laboratory techniques. Since there is no exact description the samples were processed following our standards (centrifugation at 4000rpm for 10 minutes at 10°C). Samples can be kept cool for two days at 2-8°C, for storage -70°C or below is recommended. A dilution of at least 1/10 is necessary but recommended is a dilution of 1/500. A dilution of 1/500 was used in this study.

Urine:

Collection based on standard laboratory techniques, centrifugation is recommended. Like serum the samples can be kept cool for two days at 2-8°C or be stored at -70°C or below. A dilution of 1/10 is necessary but a dilution of 1/500 is recommended. A dilution of 1/500 was used in this study.

2.2.1.2 ELISA 1 assay performance

(Quoted from the instruction sheet of the manufacturer [23])

Precision:

Intra-assay variation was determined by measuring two urine samples with known concentration and two plasma samples (no serum samples were tested, unknown dilution) with known concentration with 6-8 replicates.

Results:	Urine	A	CV%	14
	Urine	B	CV%	7
	Plasma	A	CV%	11
	Plasma	B	CV%	10

Inter-assay variation was determined by measuring two diluted urine samples and two diluted plasma samples of known concentration with 2-8 replicates in 2-4 separate assays.

Results:	Urine	A	CV%	4
	Urine	B	CV%	8
	Plasma	A	CV%	4
	Plasma	B	CV%	14

Sensitivity:

The lowest concentration detectable after measuring 20 assays was determined to be 1.4 pg/ml.

Specificity:

The monoclonal antibodies used bind to different preparations of recombinant human NGAL and are shown to give a single band at 25kDa on Westernblot analysis of a reduced post-nuclear supernatant from human neutrophils.

2.2.2 ELISA 2 Quantikine ELISA Human Lipocalin-2/NGAL Immunoassay

The Quantikine ELISA is a 4.5 hour solid-phase ELISA able to measure Lipocalin-2 in different human samples including urine and serum. Following the quantitative sandwich enzyme immunoassay technique a monoclonal antibody specific for human Lipocalin-2 is pre-coated onto a 96 well microplate. After standards, controls and samples are pipetted into the wells the present Lipocalin-2 is bound by the immobilized antibody to the wells. To remove any unbound substances the wells are washed and an enzyme-linked monoclonal antibody specific for Lipocalin-2 is added. The antibody binds to the Lipocalin-2 fixed in the wells. After all unbound antibodies are washed out again, a substrate solution is added and color develops in proportion to the amount of Lipocalin-2 bound to the wells in the first step. To end the reaction a stop solution is added and the intensity of the color can be measured with a microplate reader capable of measuring the absorbance at 450nm. All other supplies required are standard laboratory equipment. For the samples the end results are given in ng/ml.

2.2.2.1 ELISA 2 sample recommendations

(Quoted from the instruction sheet of the manufacturer [24])

Sample collection and storage conditions are recommended in general guidelines, sample stability has not been evaluated by the company.

Serum:

Collect in standard serum tubes and allow clotting for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Immediately remove serum and aliquot. Samples must be stored at < -20°C. Repeated freeze-thaw cycles should be avoided. Serum requires a 20-fold dilution. A dilution of 1/20 was used in this study.

Urine:

Collection of the first urine of the day, aseptically, into a sterile container. After centrifugation aliquot and store immediately < -20°C. Repeated freeze-thaw cycles should be avoided. Urine samples may require a dilution. Since no further dilution is suggested the urine samples were diluted 1/20, following the recommendation for the serum samples.

2.2.2.2 ELISA 2 assay performance

(Quoted from the instruction sheet of the manufacturer [24])

Precision:

Intra-assay precision was tested with three samples of known concentration twenty times on one plate.

Results:	Sample 1	CV %	3.6	standard deviation	0.041
	Sample 2	CV %	3.1	standard deviation	0.107
	Sample 3	CV %	4.4	standard deviation	0.334

Inter-assay precision was tested with three samples of known concentration in forty separate assays performed by at least three different technicians using two lots of components.

Results:	Sample 1	CV %	7.9	standard deviation	0.083
	Sample 2	CV %	6.1	standard deviation	0.204
	Sample 3	CV %	5.6	standard deviation	0.387

Sensitivity:

Forty assays were evaluated and the minimum detectable concentration of human Lipocalin-2 ranged from 0.003 to 0.040 ng/ml.

Specificity:

The assay recognizes natural and recombined human Lipocalin-2, in tests no significant cross-reactivity or interference was observed.

2.3 Assay performance evaluation

To evaluate the assay performance group A samples, serum and urine, were specified as Golden-Standard samples. Sample 1 for serum and urine was analyzed in five repetitions to calculate the CV% (Coefficient of Variation) while samples 2 to 9 were analyzed in doubles (see 3.2, Table 1 and 2). The mean CV% of all samples was compared with the CV% ranges stated in the manufacturers' instruction sheets.

2.4 Data analysis

To give an indication of the strength of the relationship all results were compared by calculating the coefficient of correlation (r). Only quantifiable data can be correlated but no categorical data, the numeric value of the coefficient ranges from +1.0 to -1.0.

The following guidelines usually apply: $r > 0$ indicates a positive relationship,

$r < 0$ indicates a negative relationship

$r = 0$ indicates no relationship

The closer the coefficients are to +1.0 or -1.0, the greater the strength of the relationship between the variables. Both sample absorbances and NGAL concentrations (in ng/ml) were calculated to compare the coefficient of correlation.

For significances in the group and assay comparisons the mean standard deviation was used to calculate the p-values with the T-Test, $p \leq 0.05$ demonstrated statistical significance.

The following guidelines were used: $p > 0.10$ “not significant”

$p \leq 0.10$ “marginally significant”

$p \leq 0.05$ “significant”

$p \leq 0.01$ “highly significant”

For the entire result significance first a test of Homogeneity of Variance after Levene was performed, followed by an ANOVA significance calculation with a Bonferroni Post Hoc Test.

3. Results

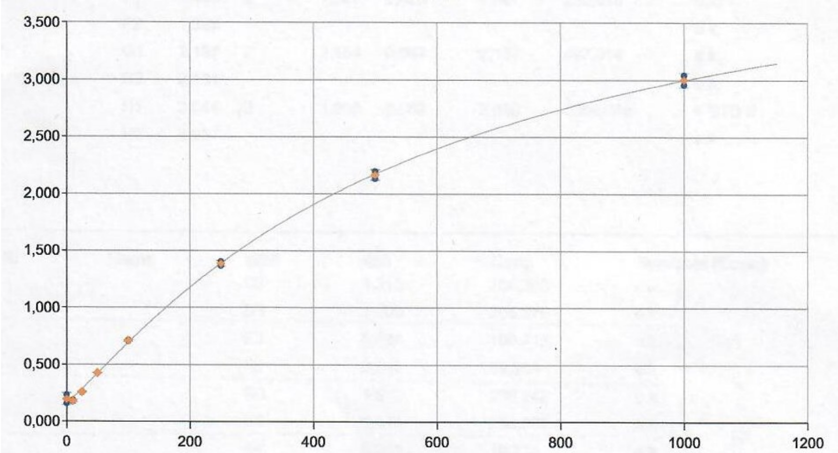
All samples of the groups A, B and C were analyzed with both assays parallel on the same day after six months of storage. Samples of groups D and E were analyzed with both assays parallel on the same day after eleven months of storage. Instructions regarding sample dilutions were followed as recommended by the companies. Only the measured absorbance at 450 is used for the inter assay calculation, since concentrations are not needed to make a comparison. For the assay comparison between ELISA 1 and ELISA 2 concentrations were calculated. The assay performance was tested by calculating CV% using a so-called golden standard sample (see 3.2). For the serum and urine results with both assays (see table 3, 5, 7 and 9) the results of group A are taken from the assay performance mean calculations (Table 1 and 2) while group B to group E were analyzed in singles because of limited microplate positions.

3.1 Standard curves

For both ELISA kits standard curves were created for each analysis. The mean absorbance of the included standards was plotted on the y-axis against the corresponding NGAL/Lipocalin-2 concentration on the x-axis.

Standard curve ELISA 1 (Figure 2) was generated using the included ready-to-use calibrators 1-8 with the known concentrations of 0, 10, 25, 50, 100, 250, 500 and 1000pg/ml. Standard curves can be used for the following test runs but it is recommended to create a curve for each run. For this study a new standard curve was created for every single run.

Standard curve for ELISA 1



Concentration pg/ml

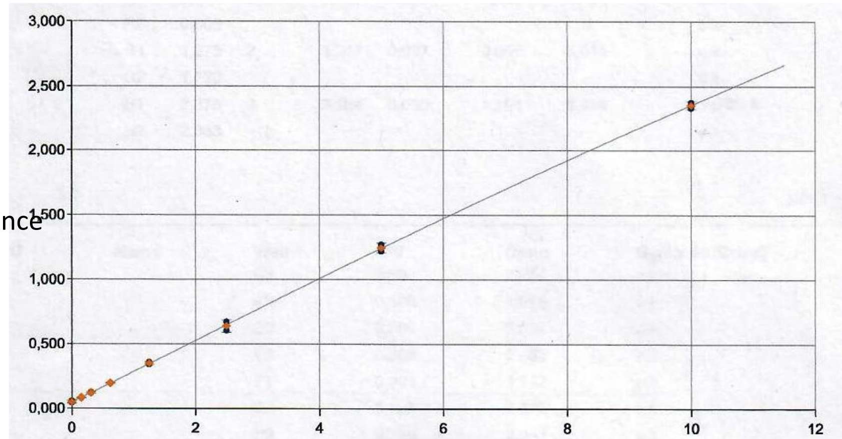
Figure 2: Standard curve for ELISA 1

Standard curve ELISA 2 (Figure 3) was generated by producing a dilution series with the included calibrator diluent. For each set of samples a standard curve was generated using standard concentrations of 0, 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10ng/ml.

Absorbance

Standard curve for ELISA 2

Absorbance



Concentration ng/ml

Figure 3: Standard curve for ELISA 2

3.2 Assay performance

Assay reliability was tested by creating so called Golden-Standard-Samples. The serum sample A1 and urine sample A1 were analyzed with both assays in a batch of five repetitions to calculate the golden standard CV%. This CV% demonstrates the reproducibility of one specific sample result within one run. The serum samples A2-A9 and urine samples A2-A9 were analyzed in doubles to calculate the CV%, like in routine where samples are always analyzed in replicates. The mean CV%, calculated from golden standard CV% and routine CV% then was compared with the intra-assay precision indicated for the assays.

The precision testing showed a good performance within or close to the expected ranges (Tables 1, 2).

Sample ID	Sample type	Result 1	Result 2	Result 3	Result 4	Result 5	Std Dev	Mean	CV (%)	Mean CV%
A 1	Serum	0.77	0.76	0.93	0.95	0.83	0.08	0.85	**9.23%**	**3.61%**
A2	Serum	1.09	1.04				0.02	1.07	**2.25%**	
A3	Serum	1.67	1.72				0.03	1.69	**1.62%**	
A4	Serum	1.33	1.21				0.06	1.27	**4.92%**	
A5	Serum	0.96	0.97				0.01	0.96	**0.73%**	
A6	Serum	0.92	1.02				0.05	0.97	**5.25%**	
A7	Serum	1.04	1.08				0.02	1.06	**1.60%**	
A8	Serum	1.87	1.97				0.05	1.92	**2.61%**	
A9	Serum	0.79	0.72				0.03	0.76	**4.30%**	
A1	Urine	0.46	0.59	0.47	0.57	0.55	0.05	0.53	**9.95%**	**8.05%**
A2	Urine	0.18	0.17				0.01	0.17	**3.74%**	
A3	Urine	0.16	0.19				0.01	0.17	**7.23%**	
A4	Urine	0.70	0.83				0.07	0.76	**8.77%**	
A5	Urine	1.63	1.54				0.05	1.58	**2.94%**	
A6	Urine	1.45	1.38				0.04	1.42	**2.79%**	
A7	Urine	1.19	1.31				0.06	1.25	**4.60%**	
A8	Urine	0.23	0.15				0.04	0.19	**20.94%**	
A9	Urine	0.79	1.00				0.10	0.90	**11.53%**	

Table 1: Results for Golden-Standard Analyses with ELISA 1

For ELISA 1 the results show a mean CV of 3.61% for serum and a mean CV of 8.05% for urine. The serum results meet the expected range of 3.6% - 4.4 % while the urine results (8.05%) are slightly out of range from the expected range of 5.6% - 7.9%.

Sample ID	Sample Type	Result 1	Result 2	Result 3	Result 4	Result 5	Std Dev	Mean	CV (%)	Mean CV%
A 1	Serum	0.68	0.64	0.71	0.60	0.64	0.04	0.65	**5.93%**	**4.38%**
A2	Serum	0.71	0.78				0.04	0.75	**4.96%**	
A3	Serum	1.00	1.13				0.06	1.06	**6.02%**	
A4	Serum	1.04	1.02				0.01	1.03	**1.26%**	
A5	Serum	0.57	0.53				0.02	0.55	**3.61%**	
A6	Serum	0.55	0.59				0.02	0.57	**3.34%**	
A7	Serum	0.71	0.74				0.02	0.72	**2.07%**	
A8	Serum	1.32	1.59				0.14	1.46	**9.27%**	
A9	Serum	0.50	0.47				0.01	0.49	**2.98%**	
A1	Urine	0.33	0.30	0.36	0.30	0.36	0.03	0.33	**8.48%**	**6.64%**
A2	Urine	0.07	0.06				0.00	0.07	**6.52%**	
A3	Urine	0.08	0.06				0.01	0.07	**10.14%**	
A4	Urine	0.35	0.39				0.02	0.37	**6.49%**	
A5	Urine	1.30	1.25				0.03	1.28	**2.16%**	
A6	Urine	1.10	1.16				0.03	1.13	**2.52%**	
A7	Urine	0.69	0.81				0.06	0.75	**8.28%**	
A8	Urine	0.07	0.05				0.01	0.06	**12.90%**	
A9	Urine	0.59	0.61				0.01	0.60	**2.25%**	

Table 2: Results for Golden-Standard Analyses with ELISA 2

The ELISA 2 results show a mean CV of 4.38% for serum and a mean CV of 6.64% for urine. The expected ranges are 10% – 11% for serum and 7% - 14% for urine; hence, the CV values are better than the expected ranges.

3.3 Results ELISA 1

3.3.1 Serum results ELISA 1

Table 3 shows the absorbance results of NGAL in all serum samples. The group results for each sample are shown in Figure 4, while the results distribution is depicted in Figure 5.

Serum Sample ID	**Group A**	**Group B**	**Group C**	**Group D**	**Group E**
1	0.85	0.90	0.85	1.07	0.69
2	1.07	1.10	1.13	1.26	1.51
3	1.69	1.62	1.60	1.77	2.15
4	1.27	1.31	1.51	1.67	1.97
5	0.96	0.78	0.88	0.94	1.31
6	0.97	0.90	1.03	no result	1.14
7	1.06	1.03	0.77	no result	1.49
8	1.92	1.92	2.07	no result	2.52
9	0.76	0.73	0.80	no result	1.26

Table 3: Individual serum sample absorbance values, ELISA 1 (in group D sample results 6 to 9 are missing due to assay failure, no absorbance was read)

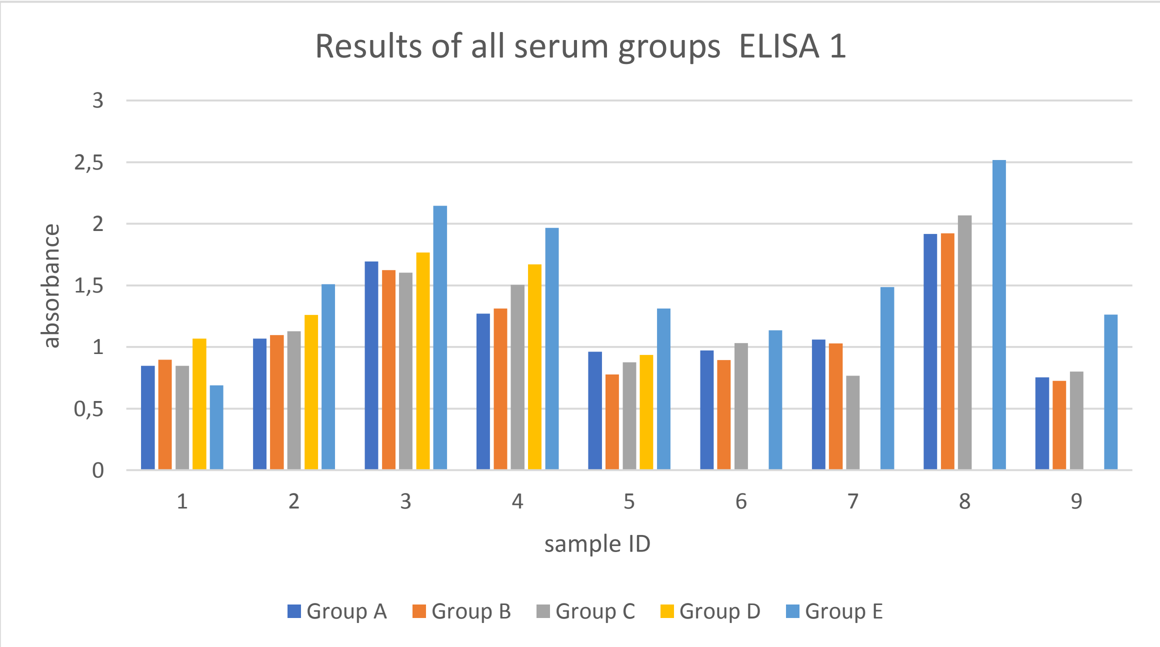


Figure 4: Serum results of all study groups, ELISA 1 (group D results of samples 6-9 are missing)

Results for samples 6 – 9 in group D are missing due to either assay failure or wrong assay handling. As depicted in Figure 4 the results show no significant tendency for different procedures, while the results for group E are higher than all the other results but for one sample. Group D, which was stored at -20°C shows better accordance than group E although company storage recommendations were -70°C.

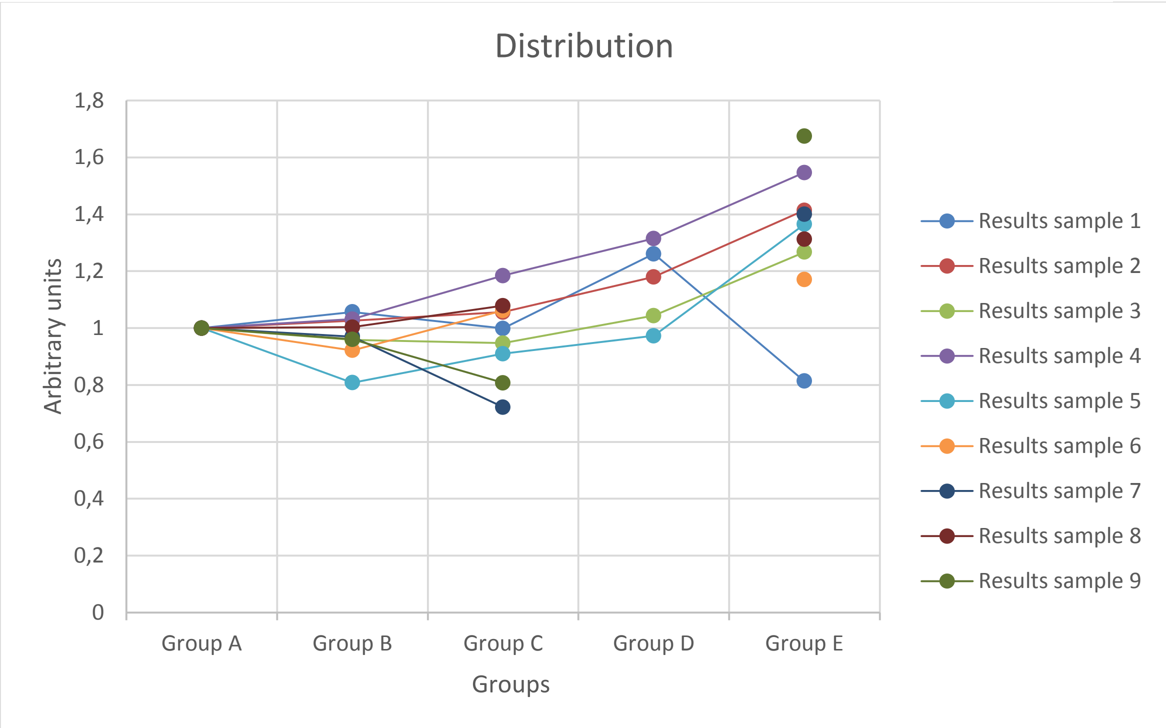


Figure 5: Normalized distribution of individual serum results, ELISA 1 (group D results of samples 6-9 are missing)

<u>Values of group A were set to 1 and all other sample values were related to this value.</u>

Serum Sample ID	Group A	Group B	Group C	Group D	Group E
1	100%	95%	100%	79%	123%
2	100%	97%	95%	85%	71%
3	100%	104%	106%	96%	79%
4	100%	97%	84%	76%	65%
5	100%	124%	110%	103%	73%
6	100%	108%	94%	no result	85%
7	100%	103%	139%	no result	71%
8	100%	100%	93%	no result	76%
9	100%	104%	94%	no result	60%
Mean		**104%**	**102%**	**88%**	**78%**
Std Dev		**8%**	**15%**	**10%**	**17%**
p- value		**0.1242**	**0.3827**	**0.0359**	**0.0037**

Table 4: Normalized distribution of individual serum results from ELISA 1 in percentage terms and p-values

Values of group A were set to 100% for each handling method. All other values were related to the value of group A.

To visualize the distribution of absorbance values, results were recalculated by dividing all the individual sample results of group B – E by the sample results of group A. In this way group A serves as reference level 1 and all other group results apply on them as reference with a value of 1. The mean values show a high accordance for group B and C and the differences are not significant. For group D the means show a difference of 12% and are significantly different while in group E we see a mean difference of 22% and highly significantly results.

3.3.2 Correlation results serum groups ELISA 1

To show differences in serum results between different procedure groups the results of groups B – E were correlated to group A, which again serves as the Golden-Standard.

<u>Results group A and group B</u> **r** = 0.98

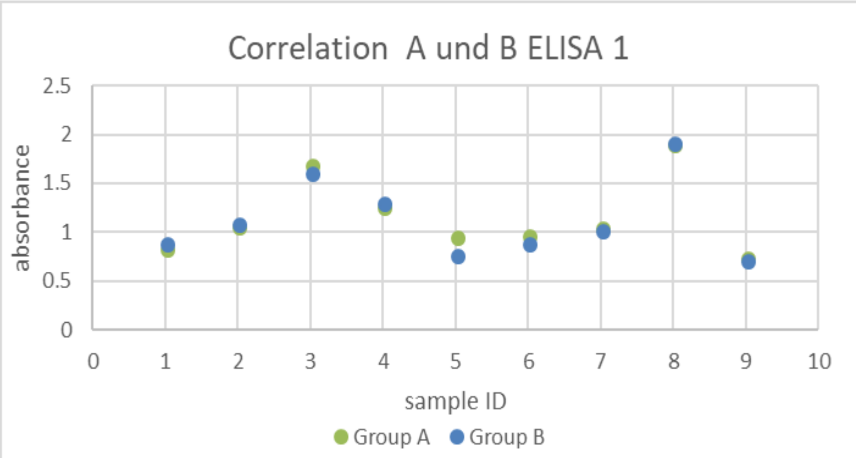


Figure 6: Correlation serum groups A and B ELISA 1

<u>Results group A and group C</u> **r** = 0.94

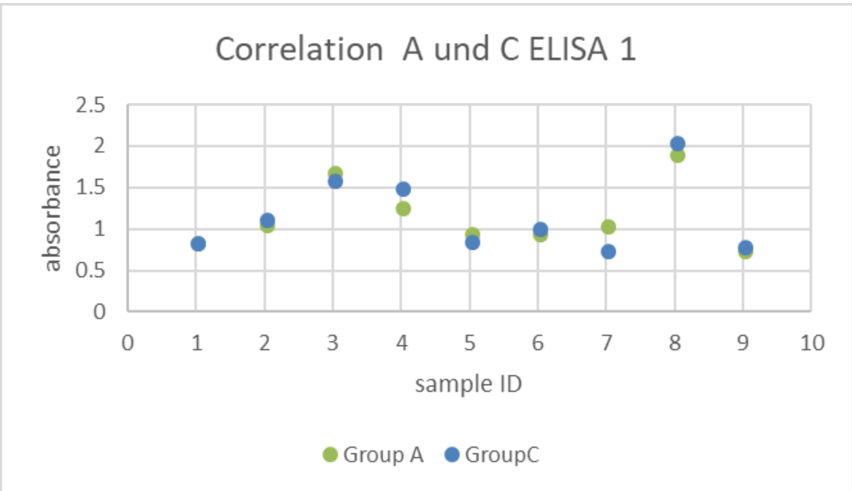


Figure 7: Correlation serum groups A and C ELISA 1

Results group A and group D   **r** = 0.89

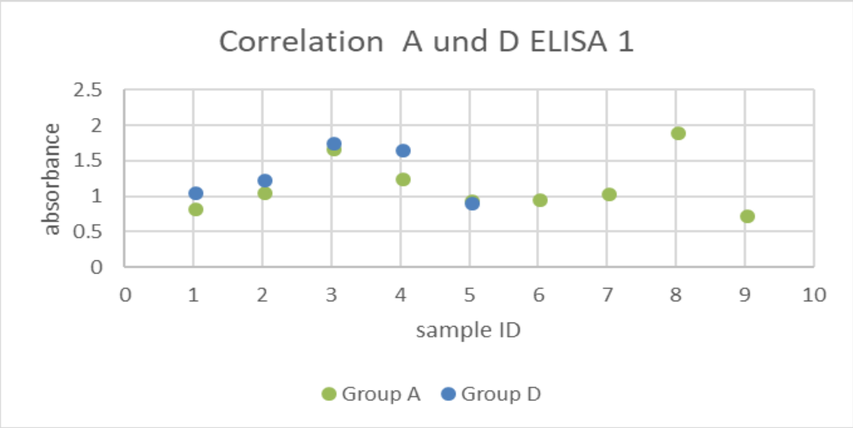


Figure 8: Correlation serum groups A and D ELISA 1 (results 6 to 9 are missing)

Results group A and group E   **r** = 0.92

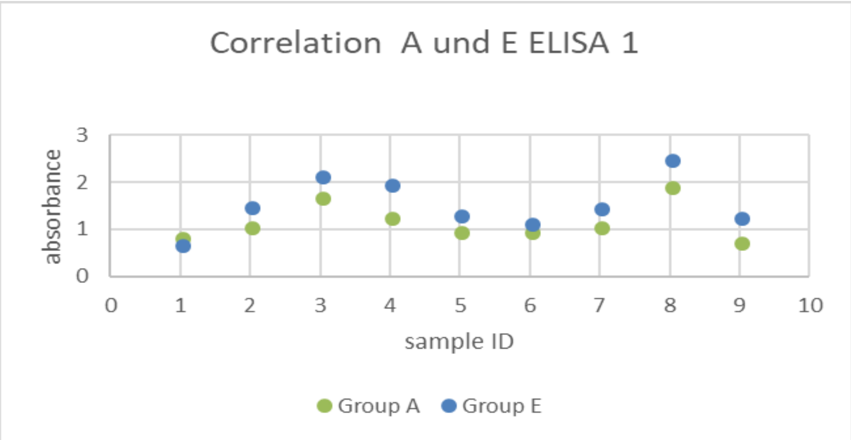


Figure 9: Correlation serum groups A and E ELISA 1

The correlation results **r** for the serum results range from 0.89 to 0.98 and show a very strong relationship between the results. The correlations A-B, A-C and A-D (Figures 6-8) show no tendencies while in Figure 9, showing the correlation A-E, it is discernible that sample values in group E are generally higher than in group A.

3.3.3 Urine results ELISA 1

Table 5 shows the absorbance results of NGAL in all urine samples. The group results for each sample are shown in Figure 10, while the results distribution is depicted in Figure 11.

Urine Sample ID	Group A	Group B	Group C	Group D	Group E
1	0.53	0.57	0.59	0.70	0.69
2	0.17	0.20	0.18	0.26	0.25
3	0.17	0.19	0.19	0.20	0.22
4	0.76	0.80	0.78	0.78	1.01
5	1.58	1.41	1.44	1.99	2.33
6	1.42	1.45	1.56	no result	2.07
7	1.25	1.05	1.34	no result	0.12
8	0.19	0.25	0.16	no result	1.68
9	0.90	0.85	1.08	no result	1.28

Table 5: Individual urine sample absorbance values, ELISA 1 (In group D sample results 6 to 9 are missing due to assay failure, no absorbance was read)

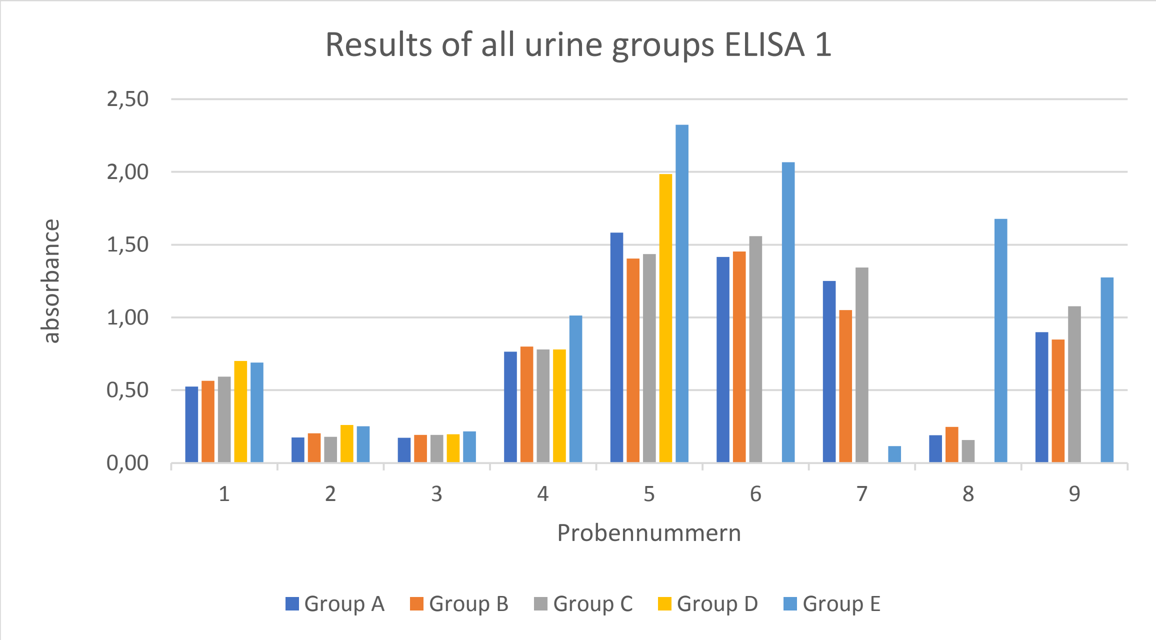


Figure 10: Urine results of all study groups, ELISA 1 (group D results of samples 6-9 are missing)

Results for samples 6 – 9 in group D are missing due to either assay failure or wrong assay handling. Following the same tendencies like the serum results the short storage urine samples (A-C) shows no considerable variation. The long storage urine samples (E-D) show noticeably different results with group E results being tendentious higher than the rest. The results for samples 7 and 8 in group E lead to a strong suspicion of being possibly switched during analyses.

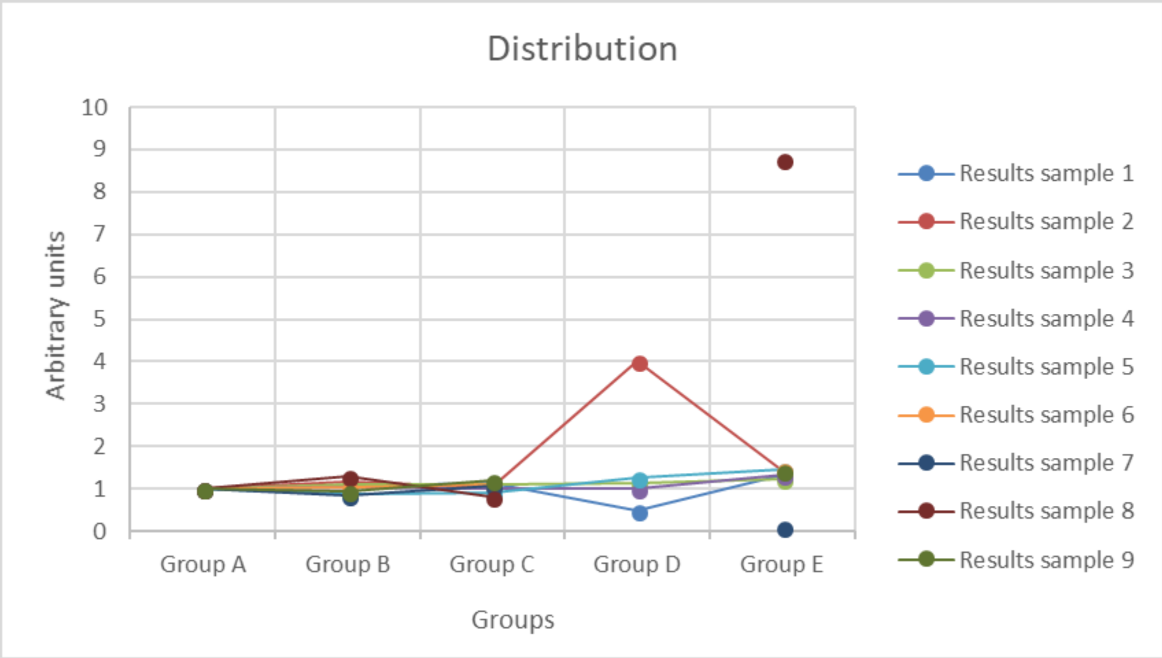


Figure 11: Normalized distribution of individual urine results, ELISA 1 (group D results of samples 6-9 are missing)

Values of group A were set to 1 and all other sample values were related to this value.

Urine Sample ID	**Group A**	**Group B**	**Group C**	**Group D**	**Group E**	**Group E ***
1	100%	93%	89%	75%	76%	76%
2	100%	85%	97%	67%	85%	85%
3	100%	90%	90%	88%	80%	80%
4	100%	96%	98%	98%	75%	75%
5	100%	113%	110%	80%	68%	68%
6	100%	97%	91%	no result	68%	68%
7	100%	119%	93%	no result	1078%	
8	100%	77%	122%	no result	11%	
9	100%	106%	83%	no result	63%	63%
Mean		**97%**	**97%**	**81%**	**178%**	**74%**
Std Dev		**12%**	**11%**	**11%**	**319%**	**7%**
p- value		**0.2804**	**0.2360**	**0.0129**	**0.2532**	**0.000047**

Table 6: Normalized distribution of individual urine results from ELISA 1 in percentage terms and p-values

Values of group A were set to 100% for each handling method. All other values were related to the value of group A. Group E was calculated including the possibly switched results of samples 7 and 8 and recalculated without these sample results, named as group E*.

To visualize the distribution of absorbance values, the results were recalculated by dividing all the individual sample results of groups B - E by the sample results of group A. In this way group A serves as a reference with a value of 1 and all other group results apply on them as reference value. As seen in the serum results, groups B and C show very high accordance with a mean of 97% and no significances found. In group D we have a mean difference of 19% and see highly significant difference. In group E two calculations were done, one with all sample results and a second excluding the possibly switched results of samples 7 and 8. While the entire calculations shows a mean difference of 78% and no significance the adapted results mirror the serum results with a mean difference of 26% and a high significance.

3.3.4 Correlation results urine groups ELISA 1

To show differences in urine results between different procedure groups the results of groups B – E were correlated to group A, which again serves as the Golden-Standard.

<u>Results group A and group B</u> **r** = 0.98

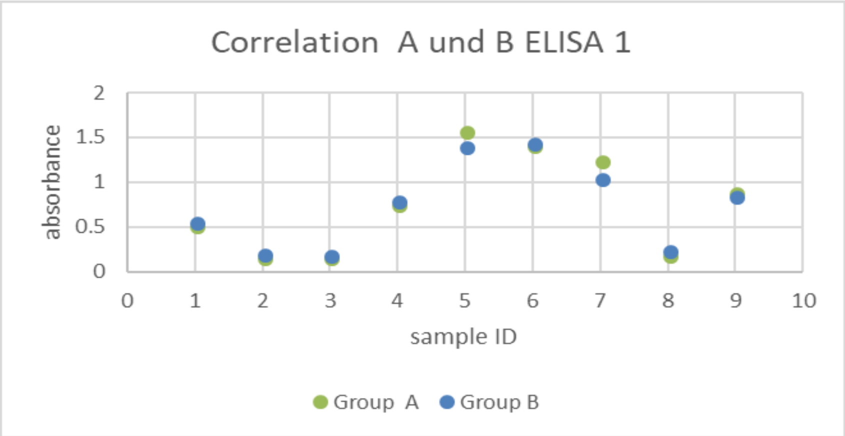


Figure 12: Correlation urine groups A and B ELISA 1

Results group A and group C **r** = 0.98

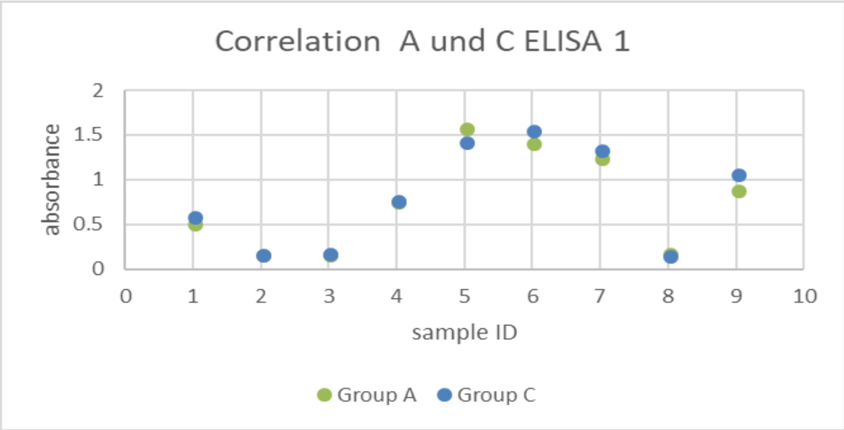


Figure 13: Correlation urine groups A and C ELISA 1

Results group A and group D **r** = 0.99

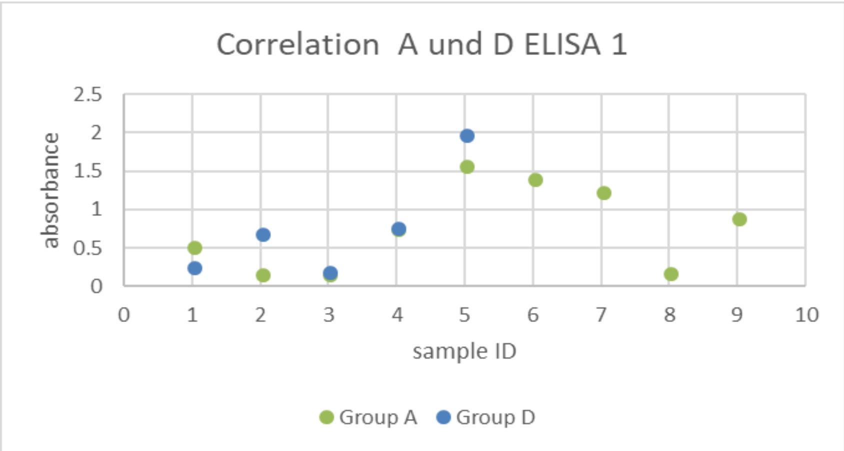


Figure 14: Correlation urine groups A and D ELISA 1 (results 6 to 9 are missing)

Results group A and group E **r** = 0.54 (recalculated with E* **r** = 0.99)

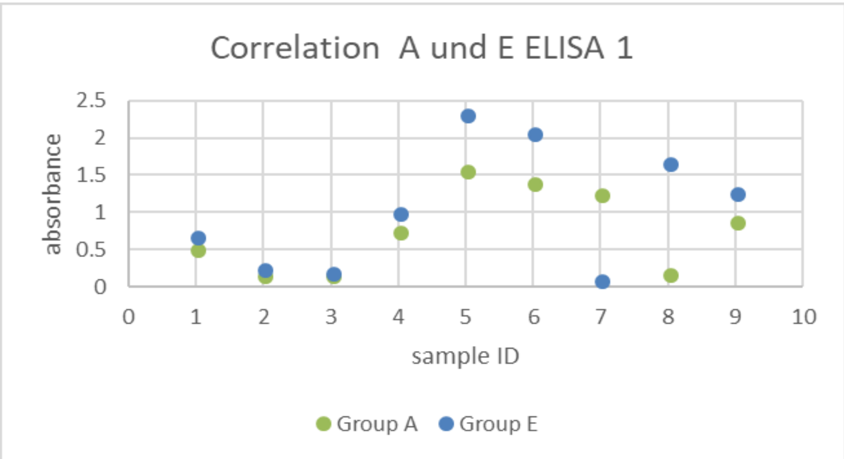


Figure 15: Correlation urine groups A and E ELISA 1

The correlation results **r** for the urine results range from 0.54 to 0.99. While the correlations A-B, A-C and A-D are 0.90 (Figure 12 to 14) or higher and show a good relationship, the correlation A-E is only 0.54 (Figure 15). The results in group E are in all samples but one higher than the results of group A and show a poor relationship. As for the distribution **r** was recalculated in group E excluding the samples 7 and 8 and changes from 0.54 to 0.99.

3.4 Results ELISA 2

3.4.1 Serum results ELISA 2

Table 7 shows the absorbance results of NGAL in all serum samples. The group results for each sample are shown in Figure 16, while the results distribution is depicted in Figure 17.

Serum					
Sample ID	**Group A**	**Group B**	**Group C**	**Group D**	**Group E**
1	0.65	0.51	0.63	0.51	0.58
2	0.75	0.80	0.71	0.55	0.77
3	1.06	1.17	1.22	0.99	1.51
4	1.03	0.96	0.95	0.86	0.90
5	0.55	0.49	0.57	0.44	0.48
6	0.57	0.56	0.58	0.51	0.77
7	0.72	0.61	0.70	0.57	0.70
8	1.46	1.42	1.42	1.38	1.70
9	0.49	0.38	0.43	0.37	0.58

Table 7: Individual serum sample absorbance values, ELISA 2

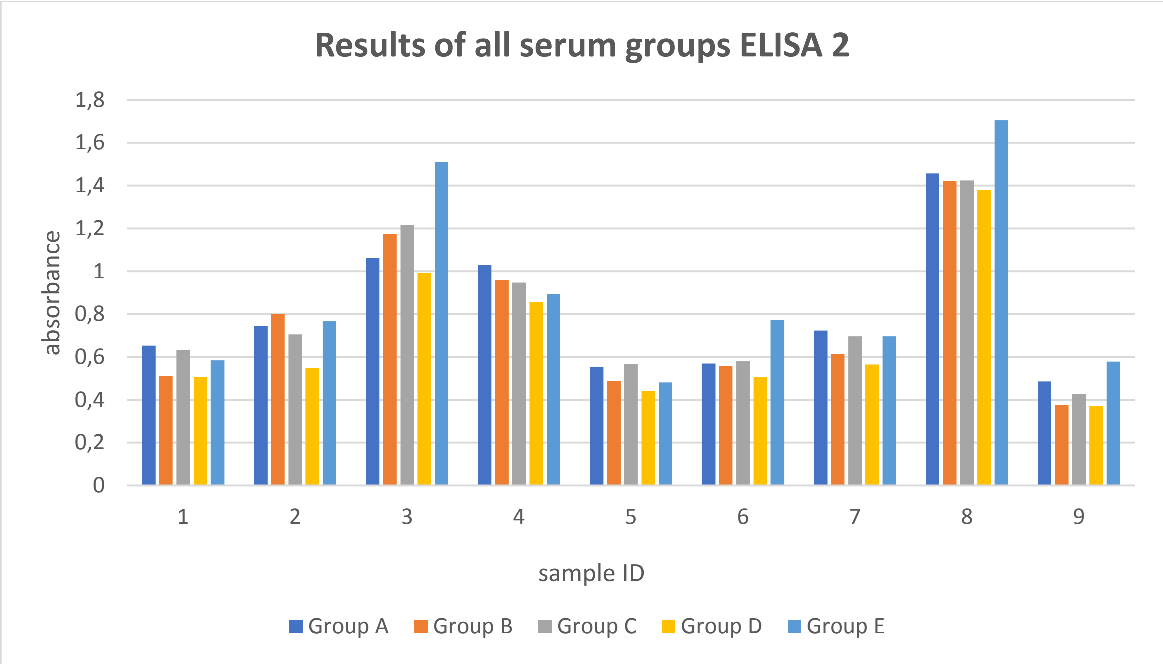


Figure 16: Serum results of all study groups, ELISA 2

ELISA 2 shows higher accordance than ELISA 1 (Figure 4). Only the results of group D show a tendency to be lower than any other group results in all serum samples.

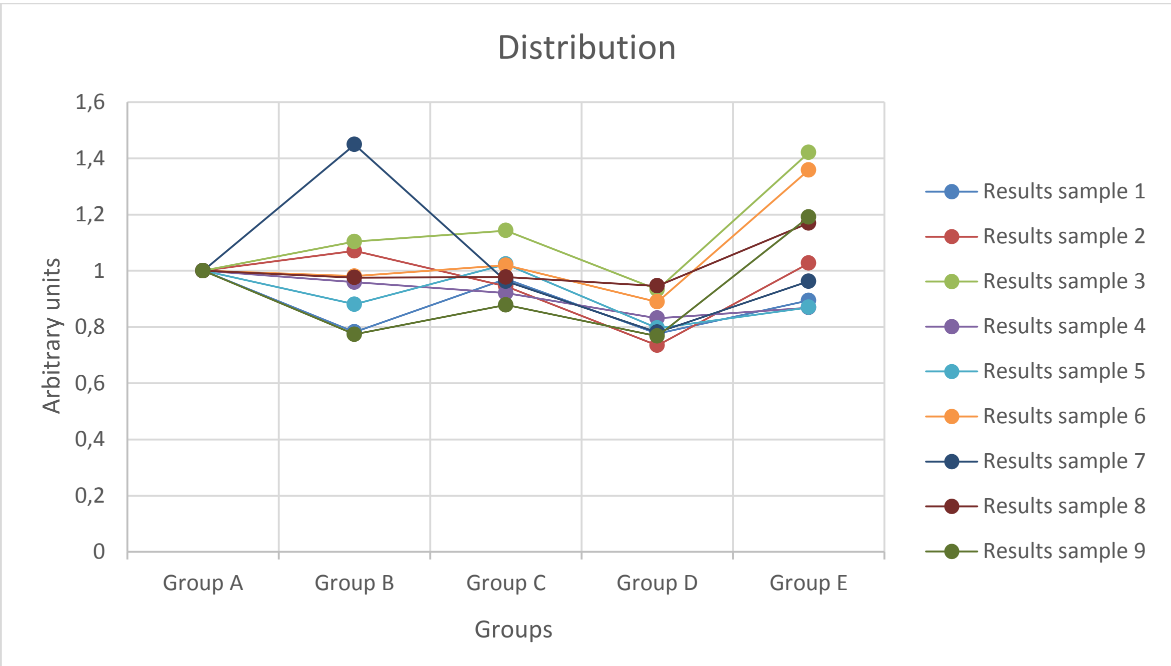


Figure 17: Normalized distribution of individual serum results, ELISA 2

Values of group A were set to 1 and all other sample values were related to this value.

Serum Sample ID	Group A	Group B	Group C	Group D	Group E
1	100%	128%	103%	129%	112%
2	100%	93%	106%	136%	97%
3	100%	91%	87%	107%	70%
4	100%	107%	109%	120%	115%
5	100%	114%	98%	126%	115%
6	100%	102%	98%	112%	74%
7	100%	118%	104%	128%	104%
8	100%	102%	102%	106%	86%
9	100%	129%	114%	130%	84%
Mean		**109%**	**102%**	**122%**	**95%**
Std Dev		**13%**	**7%**	**10%**	**16%**
p- value		**0.03863**	**0.19048**	**0.00017**	**0.21566**

Table 8: Normalized distribution of individual serum results from ELISA 2 in percentage terms and p-values

Values of group A were set to 100% for each handling method. All other values were related to the value of group A.

To visualize the distribution of absorbance values, the results were recalculated by dividing all the individual sample results of groups B – E by the sample results of group A. In this way group A serves as a reference with a value of 1 and all other group results apply on them as reference value. Groups C and E show mean differences of 2% respectively 5% and no significance. The results of group B with a mean difference of 9% are significantly higher while the results of group D with a mean difference of 22% are even highly significantly higher.

3.4.2 Correlation results serum groups ELISA 2

To show differences in serum results between different procedure groups the results of groups B – E were correlated to group A, which serves as the Golden-Standard.

<u>Results group A and group B</u> **r** = 0.97

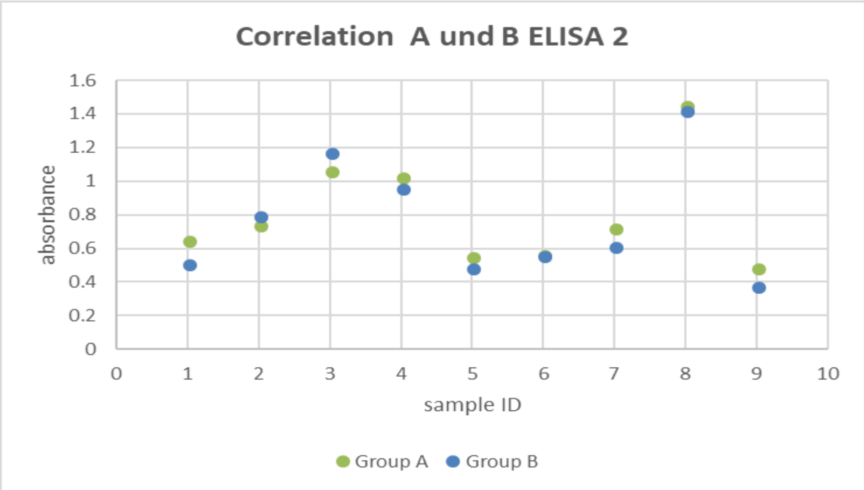


Figure 18: Correlation serum groups A and B ELISA 2

<u>Results group A and group C</u> **r** = 0.98

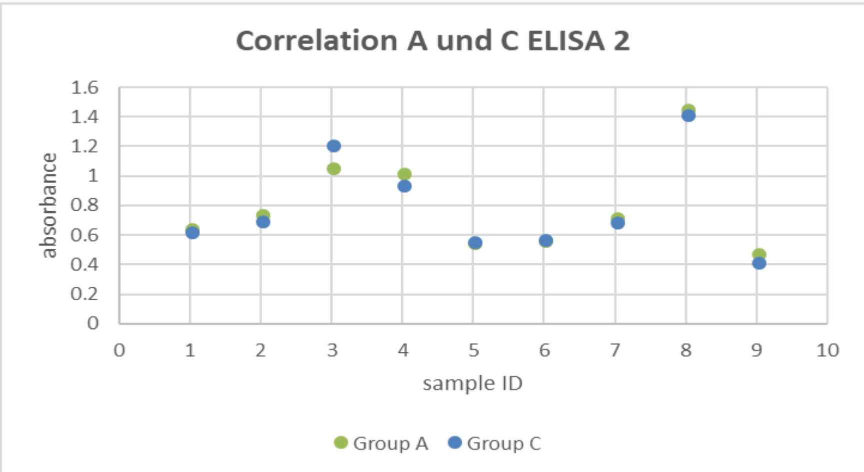


Figure 19: Correlation serum groups A and C ELISA 2

<u>Results group A and group D</u> **r** = 0.99

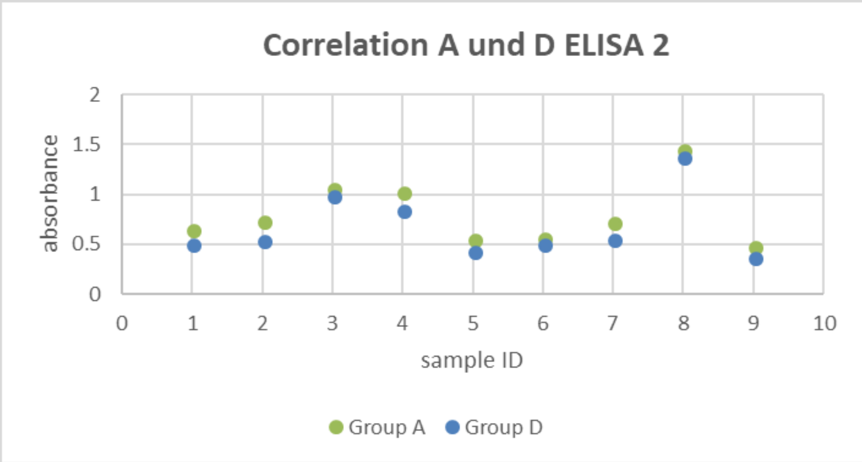


Figure 20: Correlation serum groups A and D ELISA 2

<u>Results group A and group E</u> **r** = 0.91

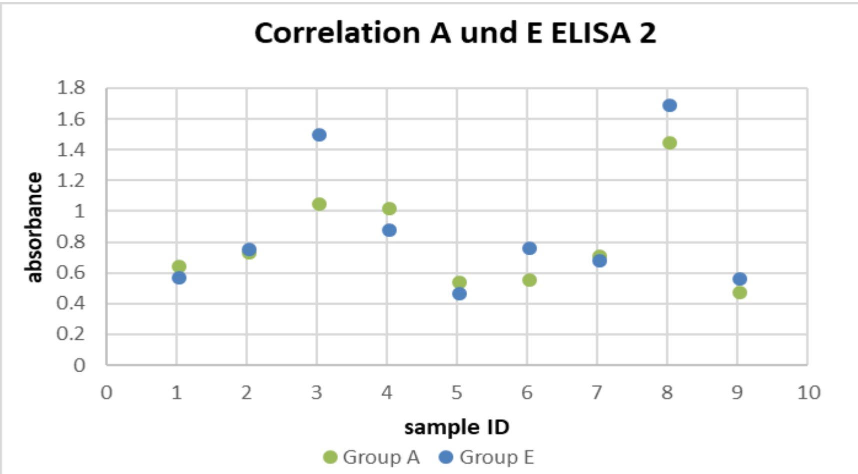


Figure 21: Correlation serum groups A and E ELISA 2

The correlation results **r** for the serum groups range from 0.91 to 0.99, corresponding with the good assay performance. The correlation A-E (Figure 21) is the lowest with 0.91 but shows no tendency for one group.

3.4.3 Urine results ELISA 2

Table 9 shows the absorbance results of NGAL in all urine samples. The group results for each sample are shown in Figure 22, while the results distribution is depicted in Figure 23.

Urine Sample ID	Group A	Group B	Group C	Group D	Group E
1	0.33	0.29	0.32	0.26	0.30
2	0.07	0.08	0.07	0.06	0.08
3	0.07	0.07	0.06	0.07	0.07
4	0.37	0.41	0.38	0.32	0.45
5	1.28	1.41	1.24	1.17	1.17
6	1.13	1.11	1.04	0.99	1.14
7	0.75	0.75	0.73	1.08	0.81
8	0.06	0.06	0.06	0.06	0.08
9	0.60	0.56	0.54	0.54	0.70

Table 9: Individual urine sample absorbance values, ELISA 2

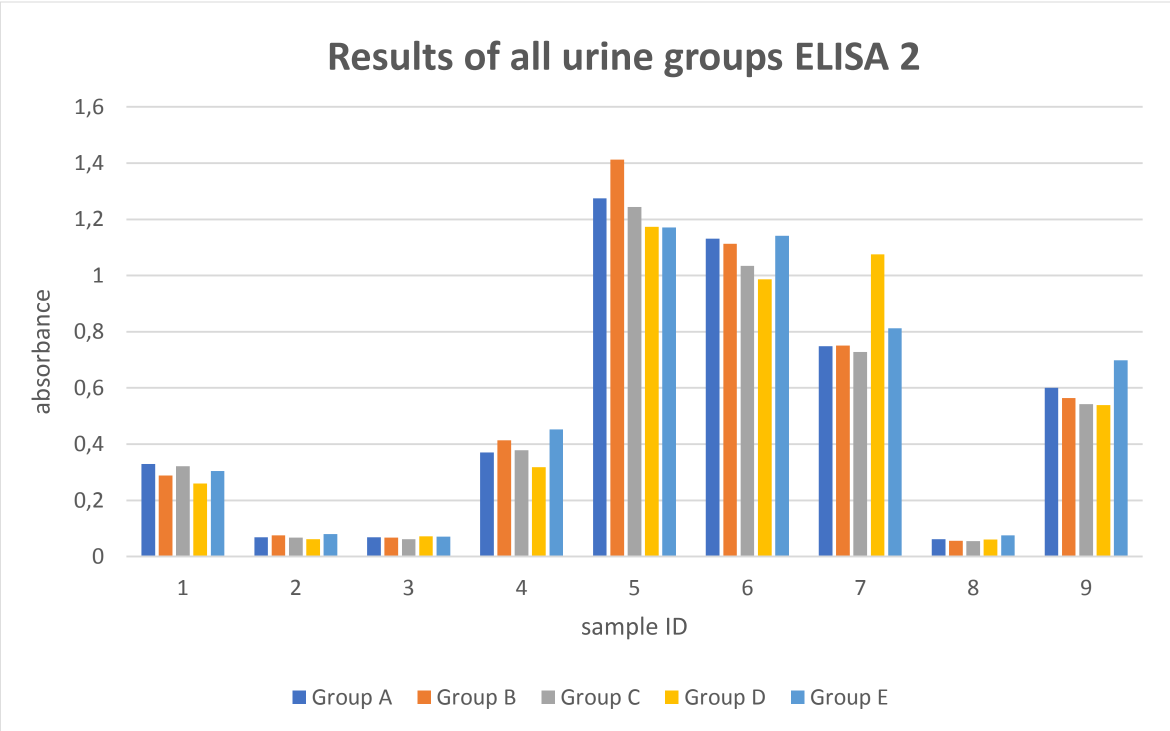


Figure 22: Urine results of all study groups, ELISA 2

All urine results are in an expected range, no group shows a significant tendency although there are a few outliners in groups B and D.

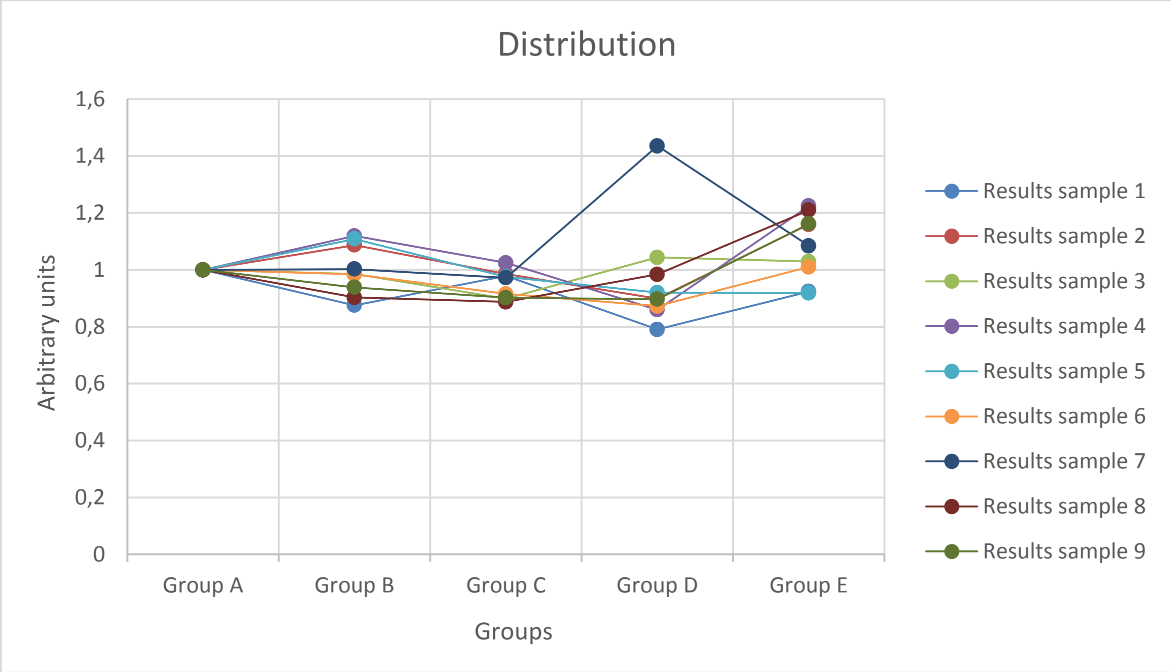


Figure 23: Normalized distribution of individual urine results, ELISA 2

Values of group A were set to 1 and all other sample values were related to this value.

Urine Sample ID	Group A	Group B	Group C	Group D	Group E
1	100%	114%	102%	127%	108%
2	100%	92%	101%	111%	86%
3	100%	101%	111%	96%	97%
4	100%	89%	98%	116%	82%
5	100%	90%	102%	109%	109%
6	100%	102%	109%	115%	99%
7	100%	100%	103%	70%	92%
8	100%	111%	113%	102%	83%
9	100%	107%	111%	112%	86%
Mean		**101%**	**106%**	**106%**	**94%**
Std Dev		**8%**	**5%**	**15%**	**10%**
p- value		**0.4148**	**0.0070**	**0.1413**	**0.0506**

Table 10: Normalized distribution of individual urine results from ELISA 2 in percentage terms and p-values

Values of group A were set to 100% for each handling method. All other values were related to the value of group A.

To visualize the distribution of absorbance values, the results were recalculated by dividing all the individual sample results of groups B - E by the sample results of group A. In this way group A serves as a reference with a value of 1 and all other group results apply on them as reference value. For the urine samples groups B and C show no significance with mean differences of 1% and 6%. Group E with a mean difference of also 6% is on the edge of significance. Group C with a mean difference of 6% displays high significance.

3.4.4 Correlation results urine groups ELISA 2

To show differences in urine results between different procedure groups the results of groups B – E were correlated to group A, which again serves as the Golden-Standard.

<u>Results group A and group B</u> **r** = 0.99

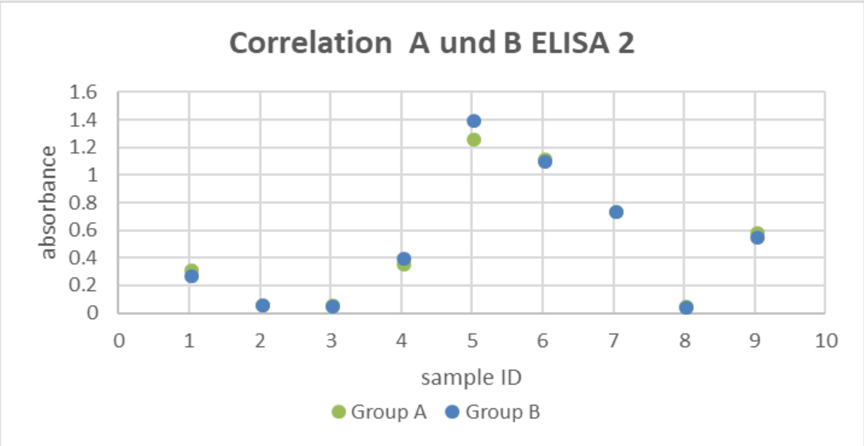


Figure 24: Correlation urine groups A and B ELISA 2

Results group A and group C **r** = 0.99

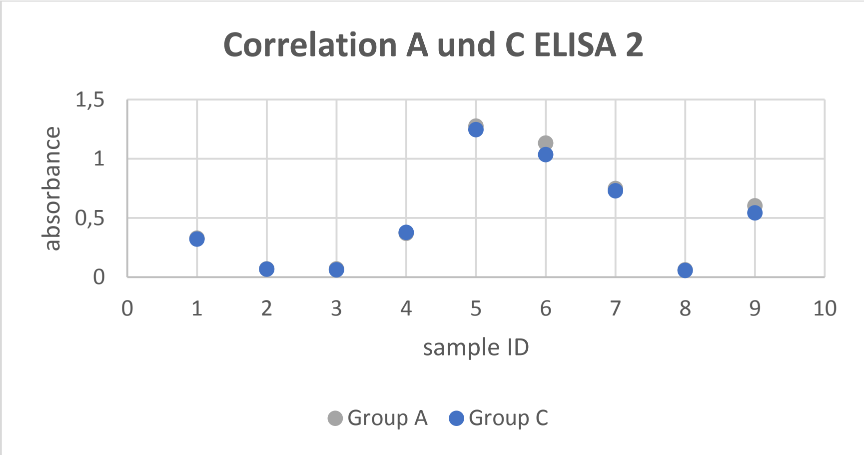


Figure 25: Correlation urine groups A and C ELISA 2

Results group A and group D **r** = 0.96

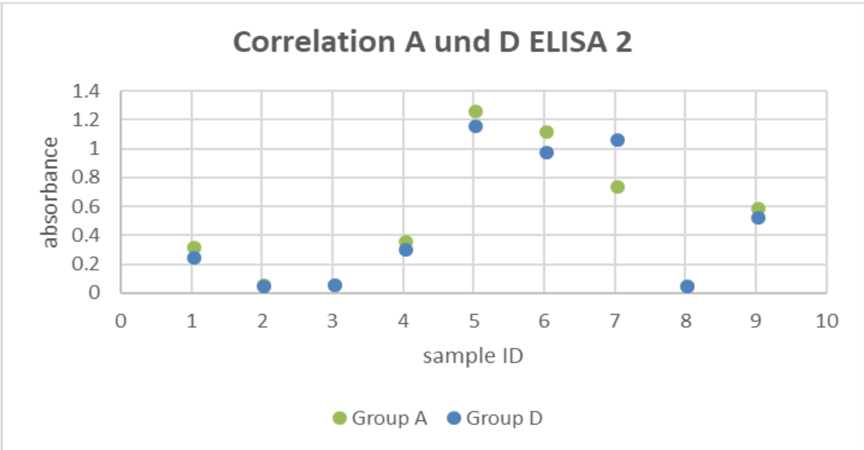


Figure 26: Correlation urine groups A and D ELISA 2

Results group A and group E **r** = 0.99

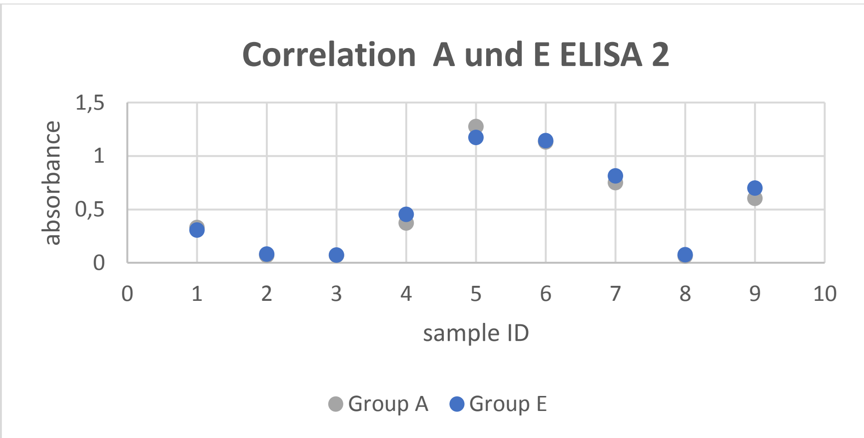


Figure 27: Correlation urine groups A and E ELISA 2

The correlation results **r** for the serum groups range from 0.96 to 0.99 and show no significant tendencies. We see an even better correlation for low absorbance samples than in the ELISA 1 results. The absorbance values of samples 1, 2, 3, 4 and 8 absorbance are nearly identical in each group correlation.

3.5 Assay comparison ELISA 1 and ELISA 2

Both assays show good intra-assay precision but to verify the results for clinical usage it is necessary to compare the two assays. The procedures are basically the same since both are sandwich ELISAS, while the manufacturers recommend different dilutions and temperature guidelines. All sample groups of the same specimen type are compared by their absorbance results and their concentration using the correlation coefficient. For the comparison calculations the concentrations of ELISA 1, which are in pg/ml, were converted to ng/ml.

3.5.1 NGAL concentrations ELISA 1 and ELISA 2

Table 11 shows the NGAL concentrations for all serum groups in ELISA 1, while Table 12 shows the concentrations for all urine groups in ELISA 1. The concentrations were multiplied with the dilution factor 500. The results are given in pg/ml in integral numbers following the recommendation of the manufacturers.

Serum										
Sample ID	Group A		Group B		Group C		Group D		Group E	
	Conc.	**pg/ml**	Conc.	**pg/ml**	Conc.	**pg/ml**	Conc.	**pg/ml**	Conc.	**pg/ml**
1	137.37	**68685**	146.97	**73485**	136.98	**68490**	176.26	**88130**	208.36	**104180**
2	184.06	**92030**	190.38	**95190**	197.74	**98870**	219.3	**109650**	298.24	**149120**
3	355.74	**177870**	332.34	**166170**	326.28	**163140**	356.22	**178110**	489.57	**244785**
4	232.51	**116255**	242.55	**121275**	296.05	**148025**	326.96	**163480**	421.77	**210885**
5	160.85	**80425**	123.01	**61505**	142.68	**71340**	128.3	**64150**	242.91	**121455**
6	162.82	**81410**	146.79	**73395**	175.8	**87900**	no result	no result	191.17	**95585**
7	182.48	**91240**	175.36	**87680**	120.86	**60430**	no result	no result	275.3	**137650**
8	439.63	**219815**	441.63	**220815**	506.04	**253020**	no result	no result	708.93	**354465**
9	118.65	**59325**	112.89	**56445**	127.56	**63780**	no result	no result	231.44	**115720**

Table 11: NGAL concentrations of all serum results, ELISA 1 (in group D sample results 6 to 9 are missing due to assay failure, no absorbance was read)

Urine										
Sample ID	Group A		Group B		Group C		Group D		Group E	
	Conc.	pg/ml	Conc.	pg/ml	Conc.	pg/ml	Conc.	pg/ml	Conc.	pg/ml
1	75.53	**37765**	82.78	**41390**	87.76	**43880**	102.04	**51020**	100.71	**50355**
2	10.18	**5090**	16.46	**8230**	11.36	**5680**	20.92	**10460**	19.21	**9605**
3	9.93	**4965**	14.05	**7025**	14.05	**7025**	8.55	**4275**	12.41	**6205**
4	120.63	**60315**	127.56	**63780**	123.61	**61805**	117	**58500**	177.55	**88775**
5	319.79	**159895**	267.6	**133800**	276.21	**138105**	428.89	**214445**	566.68	**283340**
6	270.37	**135185**	281.02	**140510**	312.27	**156135**	no result	no result	491.18	**245590**
7	227.19	**113595**	180.01	**90005**	250.91	**125455**	no result	no result	0	**0**
8	13.45	**6725**	24.78	**12390**	6.57	**3285**	no result	no result	345.77	**172885**
9	147.8	**73900**	137.38	**68690**	186.06	**93030**	no result	no result	228.79	**114395**

Table 12: NGAL concentrations of all urine results, ELISA 1 (in group D samples results 6 to 9 are missing due to assay failure, no absorbance was read)

Table 13 shows the NGAL concentrations for all serum groups in ELISA 2, while table 14 shows the concentrations for all urine groups in ELISA 2, the concentrations were multiplied with the dilution factor 20. The results are given in ng/ml in integral numbers following the recommendation of the manufacturers.

Serum										
Sample ID	Group A		Group B		Group C		Group D		Group E	
	Conc.	ng/ml	Conc.	ng/ml	Conc.	ng/ml	Conc.	ng/ml	Conc.	ng/ml
1	2.51	**50.2**	1.83	**36.6**	2.41	**48.2**	1.92	**38.4**	2.27	**45.4**
2	2.42	**48.4**	3.18	**63.6**	2.53	**50.6**	2.09	**41.8**	3.07	**61.4**
3	2.98	**59.6**	4.68	**93.6**	4.18	**83.6**	3.93	**78.6**	6.23	**124.6**
4	3.98	**79.6**	3.83	**76.6**	3.76	**75.2**	3.36	**67.2**	4.23	**84.6**
5	2.01	**40.2**	1.58	**31.6**	1.83	**36.6**	1.65	**33**	1.81	**36.2**
6	1.98	**39.6**	1.98	**39.6**	2.06	**41.2**	1.92	**38.4**	2.49	**49.8**
7	2.58	**51.6**	2.17	**43.4**	2.51	**50.2**	2.16	**43.2**	2.7	**54**
8	5.82	**116.4**	5.99	**119.8**	5.76	**115.2**	5.58	**111.6**	7.12	**142.4**
9	1.82	**36.4**	1.33	**26.6**	1.49	**29.8**	1.37	**27.4**	2.22	**44.4**

Table 13: NGAL concentrations of all serum results, ELISA 2

Urine										
Sample ID	Group A		Group B		Group C		Group D		Group E	
	Conc.	ng/ml	Conc.	ng/ml	Conc.	ng/ml	Conc.	ng/ml	Conc.	ng/ml
1	1.03	**20.6**	1.49	**29.8**	1.13	**22.6**	0.06	**1.2**	1.11	**22.2**
2	0.14	**2.8**	0.14	**2.8**	0.14	**2.8**	0.9	**18**	1.38	**27.6**
3	0.14	**2.8**	0.13	**2.6**	0.13	**2.6**	0.1	**2**	0.1	**2**
4	1.25	**25**	1.42	**28.4**	1.34	**26.8**	1.14	**22.8**	1.7	**34**
5	3.01	**60.2**	1.42	**28.4**	4.38	**87.6**	4.7	**94**	4.69	**93.8**
6	4.38	**87.6**	4.31	**86.2**	3.99	**79.8**	3.91	**78.2**	4.56	**91.2**
7	2.23	**44.6**	2.79	**55.8**	2.58	**51.6**	4.29	**85.8**	3.2	**64**
8	0.14	**2.8**	0.11	**2.2**	0.12	**2.4**	0.05	**1**	0.12	**2.4**
9	2.08	**41.6**	1.82	**36.4**	1.83	**36.6**	2.05	**41**	2.7	**54**

Table 14: NGAL concentrations of all urine results, ELISA 2

Mean values				
	ELISA 1 serum (ng/ml)	ELISA 2 serum (ng/ml)	ELISA 1 urine (ng/ml)	ELISA 2 urine (ng/ml)
Group A	109.7	58.0	66.4	32.0
Group B	106.2	59.0	62.9	30.3
Group C	112.8	59.0	70.5	34.8
Group D	120.7	53.3	67.7	38.2
Group E	170.4	71.4	107.9	43.5

Table 15: Mean values for all sample groups with ELISA 1 and ELISA 2 in ng/ml

The results show an average decrease in concentration of NGAL with ELISA 2 in all groups and samples of nearly 50%. Serum results with ELISA 2 are lower about 49% (range of 42% - 56%) and urine results about 48% (range of 40% - 56%).

3.5.2 Absorbance and concentration correlation results ELISA 1 and ELISA 2

Serum results group A

Serum						
Absorbance group A			Concentrations group A			
ELISA 1	ELISA 2	**r**	ELISA 1	ELISA 2	**r**	**p**
			ng/ml	ng/ml		
0.85	0.65	**0.95**	68.69	50	**0.86**	**0.02**
1.07	0.75		92.03	48		
1.69	1.06		177.87	60		
1.27	1.03		116.26	80		
0.96	0.55		80.43	40		
0.97	0.57		81.41	40		
1.06	0.72		91.24	52		
1.92	1.46		219.82	116		
0.76	0.49		59.33	36		

Table 16: Correlation comparison and p-value of serum group A ELISA 1 and ELISA 2 results

Urine results group A

Urine						
Absorbance group A			Concentrations group A			
ELISA 1	ELISA 2	**r**	ELISA 1	ELISA 2	**r**	**p**
			ng/ml	ng/ml		
0.53	0.33	**0.98**	37.77	20.6	**0.93**	**0.14**
0.17	0.07		5.09	2.8		
0.17	0.07		4.97	2.8		
0.76	0.37		60.32	25		
1.58	1.28		159.90	60.2		
1.42	1.13		135.19	87.6		
1.25	0.75		113.60	44.6		
0.19	0.06		6.73	2.8		
0.90	0.60		73.90	41.6		

Table 17: Correlation comparison and p-value of urine group A ELISA 1 and ELISA 2 results

Serum results group B

Serum						
Absorbance group B			Concentrations group B			
ELISA 1	ELISA 2	**r**	ELISA 1	ELISA 2	**r**	**p**
			ng/ml	ng/ml		
0.90	0.51	**0.99**	73.49	36.6	**0.98**	**0.04**
1.10	0.80		95.19	63.6		
1.62	1.17		166.17	93.6		
1.31	0.96		121.28	76.6		
0.78	0.49		61.51	31.6		
0.90	0.56		73.40	39.6		
1.03	0.61		87.68	43.4		
1.92	1.42		220.82	119.8		
0.73	0.38		56.45	26.6		

Table 18: Correlation comparison and p-value of serum group B ELISA 1 and ELISA 2 results

Urine results group B

Urine						
Absorbance group B			Concentrations group B			
ELISA 1	ELISA 2	r	ELISA 1	ELISA 2	r	p
			ng/ml	ng/ml		
0.57	0.29	**0.97**	41.39	29.80	**0.83**	**0.11**
0.20	0.08		8.23	2.80		
0.19	0.07		7.03	2.60		
0.80	0.41		63.78	28.40		
1.41	1.41		133.80	28.40		
1.45	1.11		140.51	86.20		
1.05	0.75		90.01	55.80		
0.25	0.06		12.39	2.20		
0.85	0.56		68.69	36.40		

Table 19: Correlation comparison and p-value of urine group B ELISA 1 and ELISA 2 results

Serum results group C

Serum						
Absorbance group C			Concentrations group C			
ELISA 1	ELISA 2	r	ELISA 1	ELISA 2	r	p
			ng/ml	ng/ml		
0.85	0.63	**0.95**	68.49	48.2	**0.96**	**0.03**
1.13	0.71		98.87	50.6		
1.60	1.22		163.14	83.6		
1.51	0.95		148.03	75.2		
0.88	0.57		71.34	36.6		
1.03	0.58		87.90	41.2		
0.77	0.70		60.43	50.2		
2.07	1.42		253.02	115.2		
0.80	0.43		63.78	29.8		

Table 20: Correlation comparison and p-value of serum group C ELISA 1 and ELISA 2 results

Urine results group C

Urine						
Absorbance group C			Concentrations group C			
ELISA 1	ELISA 2	r	ELISA 1	ELISA 2	r	p
			ng/ml	ng/ml		
0.59	0.32	**0.95**	43.88	22.60	**0.97**	**0.14**
0.18	0.07		5.68	2.80		
0.19	0.06		7.03	2.60		
0.78	0.38		61.81	26.80		
1.44	1.24		138.11	87.60		
1.56	1.04		156.14	79.80		
1.34	0.73		125.46	51.60		
0.16	0.06		3.29	2.40		
1.08	0.54		93.03	36.60		

Table 21: Correlation comparison and p-value of urine group C ELISA 1 and ELISA 2 results

Serum results group D

Serum						
Absorbance group D			Concentrations group D			
ELISA 1	ELISA 2	r	ELISA 1	ELISA 2	r	p
			ng/ml	ng/ml		
1.07	0.51	0.98	88.13	38.40	0.98	0.02
1.26	0.55		109.65	41.80		
1.77	0.99		178.11	78.60		
1.67	0.86		163.48	67.20		
0.94	0.44		64.15	33.00		
no result	0.51		no result	38.40		
no result	0.57		no result	43.20		
no result	1.38		no result	111.60		
no result	0.37		no result	27.40		

Table 22: Correlation comparison and p-value of serum group D ELISA 1 and ELISA 2 results

Urine results group D

Urine						
Absorbance group D			Concentrations group D			
ELISA 1	ELISA 2	r	ELISA 1	ELISA 2	r	p
			ng/ml	ng/ml		
0.26	0.06	0.99	51.02	1.20	0.95	0.37
0.70	0.26		10.46	18.00		
0.20	0.07		4.28	2.00		
0.78	0.32		58.50	22.80		
1.99	1.17		214.45	94.00		
no result	0.99		no result	78.20		
no result	1.08		no result	85.80		
no result	0.06		no result	1.00		
no result	0.54		no result	41.00		

Table 23: Correlation comparison and p-value of urine group D ELISA 1 and ELISA 2 results

Serum results group E

Serum						
Absorbance group E			Concentrations group E			
ELISA 1	ELISA 2	r	ELISA 1	ELISA 2	r	p
			ng/ml	ng/ml		
0.69	0.58	0.87	104.18	45.40	0.96	0.005
1.51	0.77		149.12	61.40		
2.15	1.51		244.79	124.60		
1.97	0.90		210.89	84.60		
1.31	0.48		121.46	36.20		
1.14	0.77		95.59	49.80		
1.49	0.70		137.65	54.00		
2.52	1.70		354.47	142.40		
1.26	0.58		115.72	44.40		

Table 24: Correlation comparison and p-value of serum group E ELISA 1 and ELISA 2 results

Urine results group E recalculated group E* (**r**=0.97 / **p**=0.09)

Urine						
Absorbance group E			Concentrations group E			
ELISA 1	ELISA 2	r	ELISA 1	ELISA 2	r	p
			ng/ml	ng/ml		
0.69	0.30	**0.60**	50.36	22.20	**0.61**	**0.17**
0.25	0.08		9.61	27.60		
0.22	0.07		6.21	2.00		
1.01	0.45		88.78	34.00		
2.33	1.17		283.34	93.80		
2.07	1.14		245.59	91.20		
0.12	0.81		0.00	64.00		
1.68	0.08		172.89	2.40		
1.28	0.70		114.40	54.00		

Table 25: Correlation comparison and p-value of urine group E ELISA 1 and ELISA 2 results

In comparison between absorbance and concentration results the coefficient of correlation (r) ranges in serum groups from 0.86 to 0.99 with a maximum difference of 0.09 in groups A and E. In the urine groups we have a range from 0.60 to 0.99 with a maximum difference of 0.14 in group B. All correlation results are in an acceptable range except urine group E with 0.60 (absorbance) and 0.61(concentration).

In groups A and B the correlation is higher in concentration for both sample types while in groups C and E the absorbance correlation is higher in both sample types. In group D the correlations are identical for serum while for urine the absorbance correlation is higher than the concentration correlation. In group E the correlation results differ between serum and urine results from a mean of 0.91 for serum to only 0.60 for urine results.

For all urine groups A to D no significance was found but for the serum groups A to D significance ranges from 0.02 to 0.04. In group E we have high significance in serum results (p-value of 0.005) and no significance for urine results. If urine results for samples 7 and 8 are excluded (Group E*) the correlation changes to 0.97 and p-value from 0.17, not significant to 0.09, marginally significant.

3.5.3 Significance analysis

<u>Homogeneity of Variances</u>

Test of Homogeneity of Variance		Levene Statistic	Significance
ELISA 1	Serum	0.801	0.533
	Urine	1.795	0.151
ELISA 2	Serum	0.753	0.562
	Urine	0.778	0.546

Table 26: Homogeneity of variances results

<u>ANOVA and Bonferroni</u>

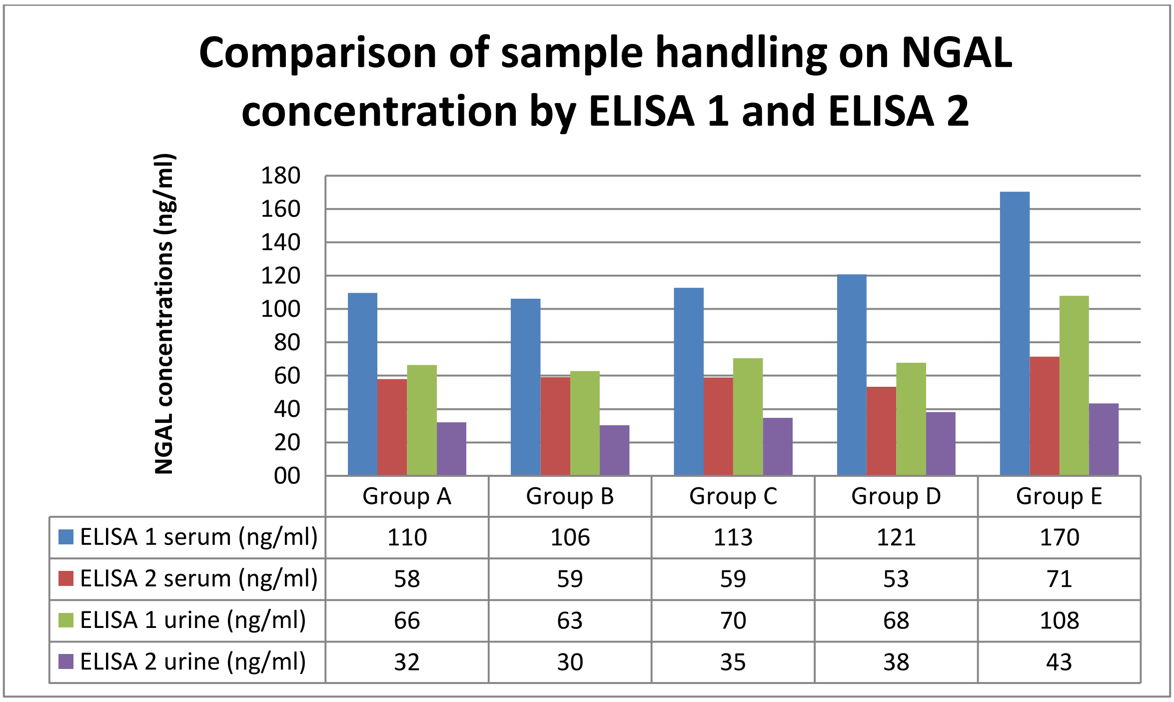


Figure 28: Comparison of sample handling on NGAL concentration ELISA 1 and ELISA 2

ANOVA		Significance
ELISA 1	Serum	0.212
	Urine	0.695
ELISA 2	Serum	0.777
	Urine	0.918

Table 27: ANOVA results ELISA 1 and ELISA 2

In the ANOVA testing with the Bonferroni post hoc test no significances were found neither within groups nor between groups.

4. Discussion

4.1 Pilot study

The pilot study was designed to investigate the influence of assay performance, sample handling and storage conditions on the results of two ELISA assays. Additionally, different variables that might occur during sample collection and storage were evaluated to give an insight of the influence of such variables on results.

Different timelines for pre-analytical procedures and storage conditions were investigated after 6 and 11 months of storage to evaluate their relevance on the measured results.

4.2 Assay performance evaluation

Both ELISA 1 and ELISA 2 are for use in research only and should not be used for clinical analysis. The assays are comparable in their handling and performance.

ELISA 1 includes only ready-to-use calibrators and reagents and is therefore easier to use for unexperienced personnel. An entire row of the microplate could not be read (absorbance=0.00), hence some results are missing because of either assay failure, like missing antibody covering of the microplate or wrong handling.

ELISA 2 needs experience and training but shows better reproducibility and with a standard concentrate to generate a standard curve, done by a dilution series for each set, gives the opportunity to adjust the standard curve if needed.

Both ELISAs show good performance, stability and practicability. The difference in NGAL concentration of about 50% reflects the different expected values. Assay performance testing was done by multiple repetitions of samples to calculate the CV%. Both assays met the ranges provided by the manufacturers. ELISA 1 serum CV 3.61% and urine CV 8.05% (slightly out of range) and ELISA 2 performing even better than expected with both sample types, serum CV 4.38% and urine CV 6.64%.

Limitations of this evaluation are the small number of samples and the fact that only one ELISA kit of each company was available for testing, so for assay failures or high CV% in sample results no measurement could be repeated. With ELISA 1 four sample results in each, serum and urine, are missing because no absorbance was read. Those samples should be reanalyzed to fill in the missing results especially concerning the already small number of samples. Also, a possible mix-up of two urine samples was detected and in a study those samples would be excluded unless identification was possible. In this pilot study the samples were used but comparison calculations were done with all results and recalculated excluding those samples.

4.3 Evaluation of results

In summary, marginally significant to highly significant results within groups in absorbance and concentration comparisons were found, but not with the following ANOVA calculation. Throughout the study correlations showed high accordance, which is displayed in homogeneity test after Levene.

In several studies, stability of NGAL and other cytokines in urine and serum was investigated. In serum samples storage at 4°C for up to 7 days before freezing was tested with the conclusion that NGAL concentration did not change significantly. Even with storage at room temperature (25°C) for up to seven days changes in concentration were noticed but were not significant [25]. While most studies use ELISA techniques for their analysis, also mass spectrometry was used to evaluate the effects of freeze and thaw cycles on the human plasma proteome. Increasing concentrations of small mass proteins in plasma were found after repeating freeze - thaw cycles, particularly after the second thawing while stability at -70°C for four years showed minimal effects. In this study, the conclusion was that degradation after long term storage has minimal effect on protein stability, while multiple freeze – thawing cycles should be avoided [26]. Other studies on NGAL in urine samples show results with only a small but marginally significant decrease in concentration after three freeze – thaw cycles, measured after only 24 hours of storage at -80°C, and a measurably but not significant decrease after storage at -80°C after five years [2].

In this pilot study, the same results were found with small differences in correlation and significant results in group comparisons but no significance after homogeneity testing and ANOVA calculation.

Why is there such an increase in NGAL concentration in serum and urine, especially with ELISA 1, after eleven months of storage at -80°C? This not verifiable. The reasons might be the biochemical effect of denaturation of the biomarker, leading to a release of the NGAL protein from a putative binding protein during storage and then leading to an increased concentration of free NGAL in the sample. Also physical effects like evaporation during storage could be an explanation, although larger changes in volume were not observed during sample analysis. A small loss in sample volume could have led to increasing protein concentrations in the samples. Increases and decreases in various proteins after long time storage at -80°C were found in a study investigating variables like gender, age or even time of sample collection. NGAL was not specifically named in this study but similar cytokines were investigated. This study implies that storage time is equally important for protein profiles as is patient history [27].

General studies about long time storage of proteins for biobanks indicate that maximum stability of macromolecules can only be ensured with storage temperatures of -150°C or lower. Storage of urine samples at higher temperatures might lead to degradation and significantly different results. Proteins in plasma and serum samples continue to denature at a freezing temperature of -80°C. Additionally, a controlled freezing rate should be maintained to prevent degradation. In research and routine facilities samples are put in freezers right after aliquoting, which leads to a very slow cooling rate of 1-2°C/ min. Also, freezing and thawing cycles should be avoided as should opening freezers repeatedly [28].

Degradation and accumulation kinetics also showed interesting results in a study based on samples stored in biobanks where samples were stored for up to 29 years at -80°C or lower. Different biomarkers that are related to one-carbon metabolism and vitamin B function were tested. Eleven markers were stable during storage, while others showed large differences in accumulation or degradation. For samples with an increase in biomarker concentration of 15% or more accumulation kinetics were determined. Those kinetics may be influenced by protein binding, precursor degradation or proteolysis [29].

General recommendations and SOPs for blood and urine samples handling procedures for biobanks were currently investigated in a study. For urine samples different centrifugation procedures were tested and showed that a mild pre-centrifugation, to spin down cellular components, followed by a centrifugation of the supernatant at a higher speed to eliminate other organic particles shows the best results. Also, annotations of time delays and temperature history is important and will add to sample quality. Urine samples should be frozen and stored at -80°C or lower [30].

In addition, blood samples were tested to investigate which anticoagulant should be used, and which sample processing and storage protocols should be followed. The investigators concluded that for plasma samples EDTA or citrate can be used, plasma being generally more stable than serum. The time delay between collection and processing should be as short as manageable, temporary storage at 4°C is recommended. Samples should be frozen immediately after processing at -80°C or lower [30].

Reliability of immunoassays has also been discussed in the last years due to the capability of mass spectrometry to detect attomoles of molecules directly. Named flaws of immunoassays are the lack of concordance, false antibody bindings because of auto-antibodies or anti-reagent antibodies and the well documented hook effect. Standardization of clinical analytes failed because standard material does not reflect the molecular population in any one individual. Genetic differences may lead to variable protein processing, modifications or cross-linking [31].

4.4 Conclusion

In conclusion, sample collection was found not to be bound to strict time and temperature guidelines, at least for NGAL. According to this pilot study, serum and urine samples can be stored at 4°C for 48 hours and therefore routine analysis can be performed before the study samples are further processed. Sample collection during night hours or weekends is possible since rest serum or urine can be used after routine laboratory analysis is finished if kept cool. Freeze-thaw cycles also showed no significant effect in neither serum nor urine samples. Storage temperatures of -20°C showed minimal effects after eleven months but is not recommended because of storage guidelines. Measurement should be finished after six months of storage at -80°C or long time storage needs to be evaluated again with samples of known concentration and patients' history information. To minimize result differences it would be possible to create a master standard curve for concentration calculation. For quality management reasons a standard curve for each run is necessary including quality controls but for concentration calculations the master standard curve could be used for all measured absorbance values. All the above conclusions are true for measurements of NGAL in human serum and urine and cannot be easily transferred to any other biomarker, whether it is a protein, a nucleic acid or a metabolite.

5. Reference list

1. Ziegler, E (2017). Studien meist wegen Betrugs zurückgezogen. [online] ORF. Available at: http://sciencev2.orf.at/stories/1705744/index.html [Accessed 29.12.2017]

2. Schuh, MP and Nehus, E and Ma, Q and Haffner, C and Bennett, M and Krawczeski, CD and Devarajan P (2016). Long-term Stability of Urinary Biomarkers of Acute Kidney Injury in Children. Am J Kidney Dis 67 (1), 50-61.

3. Wikipedia, (2017). [online] Available at: https://en.wikipedia.org/wiki/Lipocalin-2 [Accessed at 28.5.2018]

4. Chakraborty, S and Kaur, S and Guha, S and Batra, SK (2012). The multifaceted roles of neutrophil gelatinase associated lipocalin (NGAL) in inflammation and cancer. Biochem Biophys Acta 1826(1), 129-69.

5. Chan, YK and Sung, HK and Sweeney, G (2015). Iron metabolism and regulation by neutrophil gelatinase-associated lipocalin in cardiomyopathy. Clin Sci (Lond) 129(10), 851-62.

6. Bolignano, D and Coppolino, G and Romeo, A and De Paola, L and Buemi, A and Lacquaniti, A and Nicocia, G and Lombardi, L and Buemi, M (2009). Neutrophil gelatinase-associated lipocalin (NGAL) reflects iron status in haemodialysis patients. Nephrol Dial Transplant 24(11), 3398-403.

7. Barasch, J at al. (2016) [online] Disposal of iron by a mutant form of lipocalin 2. Nat. Commun 7. Available at: https://nature.com/articles/ncomms12973 [Accessed at 15.4..2018]

8. Ezzelle, J and Rodriguez-Chavez, IR and Darden, JM and Stirewalt, M and Kunwar, N and Hitchcock, R and Walter, T and D'Souza, MP (2008). Guidelines on Good Clinical Laboratory Practice: Bridging Operations between Research and Clinical Research Laboratories. J Pharm Biomed Anal. 46(1), 18-29.

9. WHO Library Cataloguing-in-Publication Data, (2012). [online] Good clinical laboratory practice (GCLP)
Available at: https://www.who.int/tdr/publications/documents/gclp-web
[Accessed at 3.12.2017]

10. EMA, (2012). [online] Reflection paper for laboratories that perform the analysis or evaluation of clinical trial samples. Available at: http://www.ema.europa.eu [Accessed at 15.12.2017]

11. Baker, M. (2016). [online] 1.500 scientists lift the lid on reproducibility. Nature 533, 452-454. Available at: http://www.nature.com/news/ [Accessed at 8.3.2018]

12.Dollfuß, H (2015). Analysis of retracted publications in the bibliographic database Web of Science between 2004 and 2014. Med Bibl Inf 15(1-2):Doc09.

13. Begley, CG and Ioannidis, J (2015). Reproducibility in Science: Improving the Standard for Basic and Preclinical Research. Circ Res. 116, 116-126.

14. Breining, G. (2017). [online] Addressing the Research Replication Crisis. Special to AAMC News. Available at: https://news.aamc.org/medical-education/article/academic-medicine-research-replication-crisis/ [Accessed at 29.12.2017]

15. Simeon-Dubach, D and Burt, AD and Hall, PA (2012). Quality really matters: the need to improve specimen quality in biomedical research. J.Pathol. 228, 431-433.

16. Jewell, S (2010). [online] Biospecimen Reporting For Improved Study Quality. Department of Pathology and Comprehensive Cancer Center The Ohio State University 1. Available at: https://www.yumpu.com/en/document/view/28035622/biospecimen-reporting-for-improved-study-quality-brisq-brn- [Accessed at 18.5.2018]

17. Medizinische Universität Innsbruck (2007). [online] Klinische Studien: Qualitätsstandards heben-myPoint. Available at: https://www.i-med.ac.at/mypoint/archiv/2007112301.xml [Accessed at 25.1.2018]

18. De Souza, Y and Greenspan, J (2013). Biobanking Past, Present and Future: Responsibilities and Benefits. AIDS. 27(3), 303-312.

19. Huppertz, B (2017). Medical University Graz, Austria. MSc in Biobanking Teaching Material 4_3 Process of sample input Part III

20. Sargsyan, K (2018). Medical University Graz, Austria. MSc in Biobanking Teaching Material 7_1 Planning and Organisation

21.Leon, A and Davis, L and Kraemer, H (2011). The Role and Interpretation of Pilot Studies in Clinical Research. J Psychiatr Res. 45(5), 626-629.

22. Lancaster ,GA and Dodd, S and Williamson, PR (2004). Design and analysis of pilot studies: recommendations for good practice. J Eval Clin Pract. 10(2), 307-12.

23.Bio Porto Instruction sheet Human NGAL ELISA kit. DK-Hellerup (2015) Available at: http://www.bioporto.com

24. R&D Systems Instruction sheet Quantikine ELISA. UK-Abingdon (2016) Available at: http://www.RnDSystems.com

25. Jing Wang, J and Hai Hong Zhu and Ji Hua Xue and Shan Shan Wu and Zhi Chen (2015). Effects of storage conditions on the stability of serum CD163, NGAL, HMGB1 and MIP2. Int J Clin Exp Pathol. 8(4), 4099-4105.

26. Mitchell, BL and Yasui, Y and Li, CL and Fitzpatrick, AL and Lampe, PD (2005). Impact of freeze-thaw cycles and storage time on plasma samples used in mass spectrometry based biomarker discovery projects. Cancer Inform. 1, 98-104

27. Enroth, S and Hallman, G and Grankvist, K and Gyllensten, U (2016). Effects of Long-Term Storage Time and Original Sampling Month on Biobank Plasma Protein Concentrations. EBioMedicine 12, 309-314.

28. ASKION (Engineering, Production, Service) (2013) [online] White paper: Langzeitlagerung von flüssigen biologischen Proben.
Available at: http://www.askion.com. [Accessed ad 24.6.2018]

29. Hustad, S and Eussen, S and Midttun, O and Ulvik, A and Van de Kant, P and Morkrid, L and Gislefoss, R and Ueland, P (2012) Kinetic Modeling of Storage Effects on Biomarker Related to B Vitamin Status and One-Carbon Metabolism. Clinical Chemistry 58(2), 402-410.

30. Bernini, P and Bertini, I and Luchinat, C and Nincheri, P and Staderini, S and Turano, P (2011). Standard operating procedures for pre-analytical handling of blood and urine for metabolomic studies and biobanks. J Biomol NMR 49, 231-243

31. Hoofnagle, A and Wener, M (2009). The Fundamental Flaws of Immunoassays and Potential Solutions Using Tandem Mass Spectrometry.
J Immuno Methods 347(1-2), 3-11.