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Analysis of pre-analytical variables for diagnostic and therapeutic applications

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A Sergio,

che amava la cultura più di ogni altra cosa.
Sarebbe stato un libro in più da aggiungere
alla tua vasta collezione.

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1 Abstract

Introduction: Pre-analytical conditions, such as patient preparation, specimen acquisition, handling and storage, can deeply influence the sample quality. The main interest of this study was thus to evaluate the impact of time, temperature and method of sampling, exemplarily conducted for pancreatic cancer serum samples and mesenchymal stromal (stem) cells (MSCs). Moreover, the applicability of storing liquid biopsies for pancreatic cancer screening was also tested. Pancreatic cancer was chosen as it remains one of the most lethal diseases since early detection is still very difficult due to two reasons: the inaccessible location of the pancreas for minimal-invasive diagnostics as well as the lack of clinical screening markers. MSCs were chosen as their clinical impact has been increasingly recognized for therapeutical applications e.g., for bone fractures.

Material & Methods: The preanalytical conditions for the two clinical applications were analysed as follows: For *pancreatic cancer*, serum samples without any storage/freezing cycle (fresh serum) were used as reference control for protein concentration analysis. A multiplex protein biochip based on a simultaneous chemiluminescent sandwich immunoassay (for C3a desArg, CD26, M-CSF and S100A11) and two ELISA kits (for M2PK and IL-18) were tested on a pool of serum samples collected from healthy donors (n = 5). Aliquots were stored at room temperature (20°C), -80°C and below -130°C. Serum samples were analysed directly without storage/freezing cycle (0h), after 3 days, 2 weeks, 3 months and 1 year of storage at the above mentioned three temperatures. Furthermore, the performance of the biochip for pancreatic cancer screening plus three additional clinical routine markers (Cyfra, CA19-9 and CEA) was evaluated for potential preanalytical impact in a multicentre validation set consisting of 431 pancreatic cancer samples and 42 healthy control serum samples, stored either below -130°C or at -80°C.

The quality of *mesenchymal stromal (stem) cells* (MSCs) was compared using three different methods of sampling: a) direct isolation and cultivation of MSCs (fresh); b) storage of whole bone fragments below -130°C with subsequent isolation and cultivation of MSCs (frozen bone) and c) cultivation of MSCs after isolation and storage below -130°C (frozen cells). The bone and the cells were frozen with a standard freezing method, comprising a medium of 10 % DMSO and 90 % FBS using a slow freezing method with Mr Frosty™ and storage below -130°C. The fresh collected cells were used as a reference for the analysis. The quality parameters (CFU, proliferation assay, differentiation ability (osteogenic & adipogenic), FACS markers) of the three sampling conditions were analysed for eight patients, comprising a total of 24 samples. Moreover, the genomic stability of these cells was tested by image cytometry-based ploidy measurement. A MSC-quality score was subsequently defined using the results of the previous part to be able to categorize the samples according to their quality parameters.

Results: The results for the pancreatic cancer samples showed that all evaluated markers had an unstable distribution when stored at 20°C ($p < 0.05$). C3a desArg and S100A11 showed significant lower expression levels after storage ($p < 0.05$), while M2PK was detected at higher levels after one year of storage ($p < 0.05$). M-CSF showed increased levels only at -80°C ($p < 0.05$), while it had a stable distribution overtime stored below -130°C. CD26 resulted in stable concentrations over the complete analysis period. IL-18 expression was at all temperature conditions below detection. When considering the multicentre study, Cyfra, CEA and CA19-9 – when analysed individually - discriminated between pancreatic cancer and healthy controls ($p < 0.05$). Considering all markers together, the combination of M-CSF, Cyfra and CA19-9 reached the best result with an area under the curve (AUC) of 0.941 (sensitivity 95 % and specificity 89.9 %). Interestingly, when considering only those samples stored below -130°C, also M-CSF and S100A11 individually differentiated between pancreatic cancer and healthy controls ($p < 0.05$). Furthermore, the AUCs of the single markers and of marker combinations also increased when considering only those samples stored below -130°C, with the combination of CD26, M-CSF, Cyfra and CA19-9 obtaining the best performance overall with an AUC of 0.987 (sensitivity 100 % and specificity 94.1 %).

The results for the MSCs samples showed that it is possible a) to isolate and directly culture MSCs from frozen bone pieces, and b) to cultivate MSCs that were frozen directly after the isolation. The sampling and storage method of MSCs have however a negative impact with a decreased proliferation rate, colony forming and differentiation abilities ($p < 0.05$), while genomic stability and marker expression of the cells does not seem to be affected. These results where used to develop a MSC-quality score that allows to categorize MSCs samples according to their quality.

Discussion: The results of both studies showed that the temperature of storage and the different sampling methods have a significant influence on the biomarkers levels and on the quality of MSCs. These preanalytical conditions could compromise analyses results and, as a consequence, not only multicentre studies but even diagnostic accuracy and therapeutic quality for individual patients. Furthermore, the importance of direct measurements of fresh, minimally pre-processed samples should not be underestimated: this approach could be implemented as a new standard in proteomic and clinical studies and therefore aid accurate biomarker discovery and validation. Additionally, sample quality assessment is significant for clinical application. For example, MSCs are highly heterogenous, even when extracted from the same tissue of origin, and this creates concerns on their applicability. Therefore, distinguishing high-quality samples from poor-quality ones, e.g., by applying the here developed MSC quality score, could help to choose which cells to use for an individual patient's therapy.

1 Zusammenfassung

Einleitung: Präanalytische Bedingungen wie Patientenvorbereitung, Probengewinnung, Handhabung und Lagerung können die Probenqualität stark beeinflussen. Das Hauptinteresse dieser Studie bestand daher darin, den Einfluss von Zeit, Temperatur und Probenahmeverfahren exemplarisch für Pankreaskrebs-Serumproben und mesenchymale Stroma-(Stamm-)Zellen (MSCs) zu bewerten. Darüber hinaus wurde auch die Anwendbarkeit der Aufbewahrung von Flüssigbiopsien für die Bauchspeicheldrüsenkrebsvorsorge getestet. Bauchspeicheldrüsenkrebs wurde ausgewählt, da er nach wie vor eine der tödlichsten Erkrankungen ist, da die Früherkennung aus zwei Gründen immer noch sehr schwierig ist: die für die minimal-invasive Diagnostik unzugängliche anatomische Lage der Bauchspeicheldrüse sowie das Fehlen klinischer Screening-Marker. MSCs wurden ausgewählt, da ihre klinische Wirkung für therapeutische Anwendungen, z. B. bei Knochenbrüchen, zunehmend anerkannt wird.

Material & Methoden: Die präanalytischen Bedingungen für die beiden klinischen Anwendungen wurden wie folgt analysiert:

Bei *Pankreaskrebs* wurden Serumproben ohne Lagerungs-/Gefrierzyklus (Frischserum) als Referenzkontrolle für die Proteinkonzentrationsanalyse verwendet. Ein Multiplex-Protein-Biochip basierend auf einem simultanen chemilumineszenten Sandwich-Immunoassay (für C3a desArg, CD26, M-CSF und S100A11) und zwei ELISA-Kits (für M2PK und IL-18) wurden an einem Pool von Serumproben von gesunden Spendern (n = 5) getestet. Aliquots wurden bei Raumtemperatur (20°C), –80°C und unter –130°C gelagert. Serumproben wurden direkt ohne Lagerungs-/Gefrierzyklus (0 h), nach 3 Tagen, 2 Wochen, 3 Monaten und 1 Jahr Lagerung bei den oben genannten drei Temperaturen analysiert. Darüber hinaus wurde der Biochip für die Bauchspeicheldrüsenkrebsvorsorge plus drei zusätzliche klinische Routinemarker (Cyfra, CA19-9 und CEA) auf potenzielle präanalytische Auswirkungen in einem multizentrischen Validierungsset bewertet, das aus 431 Bauchspeicheldrüsenkrebsproben und 42 gesunden Kontrollserumproben bestand, die entweder unter –130°C oder bei -80°C gelagert worden waren.

Die Qualität von *mesenchymalen Stroma-(Stamm-)Zellen (MSCs)* wurde mit drei verschiedenen Probenahmemethoden verglichen: a) direkte Isolierung und Kultivierung von MSCs (frisch); b) Lagerung ganzer Knochenfragmente unter –130°C mit anschließender Isolierung und Kultivierung von MSCs (gefrorener Knochen) und c) Kultivierung von MSCs nach Isolierung und Lagerung in unter –130°C (gefrorene Zellen). Die Knochen und die Zellen wurden mit einem Standard-Gefrierverfahren eingefroren, das ein Medium aus 10 % DMSO und 90 % FBS und ein langsames Gefrierverfahren mit Mr Frosty™ und Lagerung unter –130°C umfasste. Die frisch gesammelten Zellen wurden als Referenz für die Analyse verwendet. Bei acht Patienten mit insgesamt 24 Proben wurden die Qualitätsparameter

(CFU, Proliferationsassay, Differenzierungsfähigkeit (osteogen & adipogen), FACS-Marker) der drei Probenahmebedingungen analysiert. Darüber hinaus wurde die genomische Stabilität dieser Zellen durch Feulgen-Färbung und Bildzytometrie-basierte Ploidiemessung getestet. Anschließend wurde basierend auf den Ergebnissen ein MSC-Quality-Score entwickelt, um die Proben gemäß ihrer Qualität kategorisieren zu können.

Ergebnisse: Die Ergebnisse für die Pankreaskrebsproben zeigten, dass alle ausgewerteten Marker eine instabile Verteilung aufwiesen, wenn sie bei 20°C gelagert wurden ($p < 0,05$). C3a desArg und S100A11 zeigten nach Lagerung signifikant niedrigere Expressionsniveaus ($p < 0,05$), während M2PK nach einem Jahr Lagerung in höheren Konzentrationen nachgewiesen wurde ($p < 0,05$). M-CSF zeigte nur bei -80°C erhöhte Konzentrationen ($p < 0,05$), während es bei Lagerung unter –130°C eine stabile Verteilung über die Zeit aufwies. CD26 führte über den gesamten Analysezeitraum zu stabilen Konzentrationen. Die IL-18-Expression war bei allen Temperaturbedingungen unterhalb der Nachweisgrenze. Unter Berücksichtigung der multizentrischen Studie unterschieden Cyfra, CEA und CA19-9 einzeln analysiert zwischen Pankreaskrebs und gesunden Kontrollen ($p < 0,05$). Betrachtet man alle Marker zusammen, erzielte die Kombination aus M-CSF, Cyfra und CA19-9 mit einer *area under the curve* (AUC) von 0,941 das beste Ergebnis (Sensitivität 95 % und Spezifität 89,9 %). Interessanterweise differenzierten auch M-CSF und S100A11 individuell zwischen Pankreaskrebs und gesunden Kontrollen, wenn nur die in Stickstoff gelagerten Proben betrachtet wurden ($p < 0,05$). Darüber hinaus stiegen die AUCs der Einzelmarker und der Markerkombinationen auch an, wenn nur stickstoffgelagerte Proben betrachtet wurden, wobei die Kombination aus CD26, M-CSF, Cyfra und CA19-9 mit einer AUC von 0,987 (Sensitivität 100 % und Spezifität 94,1 %) das beste Ergebnis erzielte.

Die Ergebnisse für die MSCs-Proben zeigten, dass es möglich ist, MSCs aus gefrorenen Knochenstücken zu isolieren und direkt zu kultivieren, und dass es auch möglich ist, MSCs zu kultivieren, die direkt nach der Isolierung eingefroren wurden. Die Probenahme- und Lagerungsmethode von MSCs hat jedoch einen negativen Einfluss auf ihre Proliferationsrate, Koloniebildungsfähigkeit und Differenzierungsfähigkeit, während sie ihre genomische Stabilität und Markerexpression nicht zu beeinflussen scheinen. Darüber hinaus war es möglich, einen MSC-Qualitäts-Score zu erstellen, um die MSC-Proben nach ihren Qualitätsparametern zu kategorisieren.

Diskussion: Die Ergebnisse beider Studien zeigten, dass die Lagertemperatur und die unterschiedlichen Probenahmemethoden einen signifikanten Einfluss auf die Biomarkerspiegel und auf die Qualität von MSCs haben. Diese präanalytischen Bedingungen könnten Analyseergebnisse und damit nicht nur multizentrische Studien, sondern sogar die diagnostische Genauigkeit und therapeutische Qualität für einzelne Patienten beeinträchtigen. Darüber hinaus sollte die Bedeutung direkter Messungen an frischen, minimal vorverarbeiteten Proben nicht unterschätzt werden: Dieser Ansatz könnte als neuer Standard in der Proteomik und in klinischen Studien implementiert werden und somit die genaue

Entdeckung und Validierung von Biomarkern unterstützen. Darüber hinaus ist die Beurteilung der Probenqualität für die klinische Anwendung von Bedeutung. Zum Beispiel sind MSCs sehr heterogen, selbst wenn sie aus demselben Ursprungsgewebe extrahiert werden, und dies wirft einige Fragen bezüglich ihrer Anwendbarkeit auf. Daher könnte die Unterscheidung qualitativ hochwertiger von minderwertigen Proben, z. B. durch die Anwendung der hier entwickelten MSC-Qualitäts-Scores, bei der Auswahl der Zellen helfen, die für die Therapie eines einzelnen Patienten verwendet werden sollen.

2 Introduction

2.1 Pre-analytical Conditions

The term pre-analytical is defined as anything that comes before the analysis phase[2,3]. Thus, pre-analytical handling refers basically to all processes that occur after a biosample is collected and also after it is removed from storage until its analysis. Commonly, biosamples are collected and then stored for use at a later time and possibly at a different location. Biosamples include tissues, cells, and body fluids (and their constituent macromolecules and analytes thereof) and their critical biological properties must be preserved during processing, transport, and storage[4]. For this purpose, the International Society for Biological and Environmental Repositories (ISBER), the National Cancer Institute (NCI), and other organizations have drafted best practices for collection and storage of human samples[5,6]. The publication of ISO (International Organization for Standardization) 20387 (ISO 20387:2018 "Biobanking—General requirements for biobanking") can be considered an important milestone for the harmonization of biobanking procedures at international level. The general purpose of the ISO 20387:2018 guideline is to enable the use of biological material that guarantees reproducibility and comparability of scientific research results by standardizing the biosamples' life cycle stages. Its point-by-point indications provide specific tools related to policies, processes, and procedures covering the life cycle of biological materials and their associated data from collection to storage, reception and distribution, transport and traceability, preparation and preservation of biological samples, quality control (QC) of processes, and method validation and verification[7]. Other guidelines comprise the ISO/TS 20658:2017, which specifies requirements and good practice recommendations for the collection, transport, receipt and handling of samples intended for medical laboratory examinations, and the ISO15189:2012, which specifies requirements for quality and competence in medical laboratories. Furthermore, standard operation procedures for collection and storage of either fixed or unfixed human samples have been suggested, however these are rapidly evolving as more data are available [8–10]. Indeed, the majority of all errors within the diagnostic process occur in the pre-analytical processing of samples due to a lack of standardized procedures for patient preparation, specimen acquisition, handling and storage[11–13]. Pre-analytical variables can introduce in vitro modifications, either systematically or randomly, that adversely affect results. Therefore, assessing and controlling the pre-analytical handling of biospecimens is fundamental for their optimal future use, since the quality of the study will depend on the integrity of the samples[2,14]. Hence, evidence-based biobanking standards, procedures and quality controls should be implemented as they can help to ensure bio-specimens integrity and prevent degradation[15]. Pre-analytical effects can be subdivided on the basis of being either technical or biological. The former arise from effects of sample

collection, processing and storage, while the latter are inherent properties of the provider of the sample such as age and gender[16]. Furthermore, when considering a group of patients, a number of non-disease-related biological effects will also be superimposed and influence the proteome profile. These can be defined as intrinsic influences such as gender, age, ethnicity, and menopausal status and extrinsic influences which include diet (type and status), smoking/alcohol consumption, exercise, posture, medication etc. Although many intrinsic factors such as gender are 'fixed', some are more variable such as circadian rhythms and can be standardized to some degree[16]. Other important variables for sampling procedures are related to:

- the selection of blood collection tubes and anticoagulants;
- variations in clotting time or time lag before centrifugation;
- haemolysis;
- centrifugation speed and time;
- storage temperature;
- repeated freeze/thaw cycles[17].

The time between blood withdrawal and processing (centrifugation) must be kept as short as possible. Also the time delay until storage, storage temperature and time until analysis all contribute to the process of gathering a reliable set of data for statistical analysis[18,19]. In this context, different procedures have been applied over the years in order to avoid incorrect handling and storage of biospecimens. Implementation of the SPREC code (Standard PRE-analytical Code) and the BRISQ (Bio-specimen Reporting for Improved Study Quality) have been recommended. The SPREC code was specifically devised for primary samples (at the point of collection) and simple derivatives (e.g., plasma, buffy coat) and is intended to facilitate research collaborations across different laboratories and institutions handling similar specimens[20–22]. The BRISQ recommendations are intended to apply to any study in which human biospecimens (e.g., solid tissues and body fluids) are used and are an initial step towards defining reporting recommendations[23]. The BRISQ list could be applied in translational science, biomarker discovery, clinical trials, technology development, and diagnostic-assay and therapeutics development[23]. Most recently, new ISO standards were also published for the handling, documentation and processing of different biosamples, such as ISO 23118:2021 and ISO 17626:2021.

2.1.1 Biomarker Research

As defined in 1998 by the National Institutes of Health (NIH) Biomarker Working Group, a biomarker is "a characteristic objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention"[24,25]. A joint venture

on chemical safety, the International Program on Chemical Safety, led by the World Health Organization (WHO) and in coordination with the United Nations and the International Labor Organization, has defined a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease"[26]. Biomarkers can be divided into three categories: diagnostic, prognostic and predictive. A biomarker seldom belongs only to one category, and many can have multiple functions[1] (**Figure 2.1**). A biomarker that detects a disease or quantifies its extent is a possible diagnostic marker. Ideally, a diagnostic marker would be non-invasive, inexpensive, and effective to identify – in the domain of oncology - high-risk premalignant lesions. A prognostic marker provides information on, e.g., the likely course of a cancer disease in an untreated individual. A predictive marker, finally, indicates the groups of patients who probably will benefit the most from a given therapy. With predictive biomarkers, it should be possible to select the therapy with the highest likelihood of efficacy to the individual patient. Therefore, predictive markers are the basis for tailored treatment[1,27]. Although tumour tissue is the gold standard for clinical and investigational analyses, tissue biopsies are not without adverse risk, provide only a snapshot in time and location, and are limited in understanding tumour dynamics[28]. Furthermore, recent studies suggest that tumour biopsies may vastly underrepresent the heterogeneity of resistance in a single patient[29–31] and may also have profound implications for drug therapy in cancer[32]. To overcome these limitations, the idea of liquid biopsies was developed as a new way to allow non-invasive serial assessment of biomarkers which captures intra- and inter-tumoural heterogeneity. Liquid biopsy, defined as the capture of tumour-related biomarkers in a fluid sample (e.g. blood, saliva, urine, cerebrospinal fluid and other body fluids) has been extensively studied and is growing in popularity due to its minimal invasiveness, low consumption of reagent, and ease-of-use[33]. Furthermore, liquid biopsies might reflect the overall genetic and phenotypic tumoral landscape in an individual cancer patient. Thus, liquid biopsies feature the great potential to discover tumour-associated markers, especially for early detection and before clinical manifestation of the malignancy. Human serum is generally the most valuable specimen for identifying biomarkers of disease[34,35]. Serum generates from the clotting of blood, which is associated with the activation of protease cascades of the coagulation, thrombolysis, and complement systems[36]. Blood constantly perfuses most tissues of the human body and the serum carries not only blood-specific proteins but also proteins that are synthesized, secreted, or shed from cells throughout the body[35]. Since serum is readily accessible, biomarkers that are identified in serum can serve as diagnostic tests and as targets for therapeutic intervention[37]. Molecular diagnosis is increasingly used in detecting predisposed disease risk, monitoring disease progression, and identifying the patient's differential response to potential therapies. However, the important role that biomarkers also play in assessing the sample quality should not be forgotten. Many factors may influence the diagnostic results, especially regarding RNA

and protein assays, including the methods of sample collection, handling, processing, and preservation[38]. The major impediment to progress in the validation of new biomarkers is indeed the lack of standardization in how specimens are collected, handled, and stored. It is known that at least 60% of errors occurring in routine clinical chemistry laboratories are due to mistakes made in the preanalytical phase[39]. Most of them are attributable to mishandling procedures during collection, handling, preparing, or storing the specimens. Sample integrity is of utmost importance in order to make strong conclusions from the data, from the point of collection, through handling and shipment, storage and sample management and to ensure efficient retrieval of the correct sample and ability to link with relevant clinical data[40]. For these purposes, the availability of high-quality assured biobanks of body fluids using state-of-the-art methodologies with respect to sample collection, processing, and storage becomes extremely important. The quality of the operating procedures with which the biospecimens are handled directly determines the quality of the biomarker result and its ultimate applicability to the patient. Therefore, careful and thorough decision making before starting a collection is instrumental for getting the desired quality of samples[41].

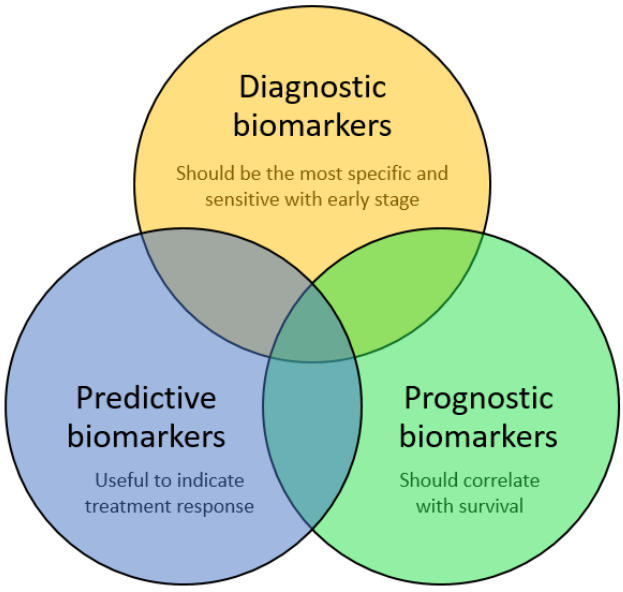

Figure 2.1 Summary of the relationships among predictive, prognostic and diagnostic markers adapted from Le et al[1].

2.1.2 Role of Biobanks in Translational Clinical Research

The word “biobank” was used for the first time in a 1996 paper investigating the role of oxidative DNA damage as an independent risk factor in cancer and was referring to the use of human biological material[42]. Since then, biobanks improved and developed in the field, thanks also to the advent of omics science (genomics, transcriptomics, proteomics, metabolomics) and the ability to develop large electronic databases that store huge amounts of information associated with patients’ clinical

phenotype data. The Organization for Economic Cooperation and Development defined biobanks as structured resources that can be used for the purpose of genetic research, including human biological materials and/or information generated from genetic analysis and associated information[43]. The European Commission published a comprehensive document highlighting the primary roles of a biobank[44]:

i) to collect and store biological materials annotated with medical data and often epidemiological data;
ii) to not consider collection projects static but continuous or long term;
iii) to associate with current and/or future research projects at the time of specimen collection;
iv) to apply coding or anonymization to assure donor privacy, along with a re-identifiable process for specific conditions where clinically relevant information becomes known and can be provided to the patient;
v) to include established governance structures and procedures (e.g., consent) that protect donors' rights and stakeholder interests.

Biobanks have a primary role in the era of precision medicine, which is based on analysing samples with clinical data[7]. The availability of a large collection of patient samples (with well-annotated patient clinical and pathological data) is a critical requirement for personalized medicine. If more high-quality samples are available through biobanks, researchers will be able to use these resources to advance patient treatment[45]. In parallel with improvements in sample management, data collection, and the increased use of biological samples for research purposes, it has become necessary to protect the patients and fulfil all the requirements of privacy, confidentiality, and human subject protection during sample sharing[7]. Finally, the extensive network established by a biobank, comprising of government and public stakeholders, helps bring together scientists, clinicians, academic researchers, the pharmaceutical industry and patient advocacy research groups, resulting into a stronger collaborative milieu in the scientific community[46].

2.1.3 Long-term Storage

The most common method of preserving biospecimens is storage of samples at low temperatures in order to inhibit degradation. Other stabilization methods include chemical fixation, plastination, drying, lyophilisation, ionic liquids, dry state storage and confinement[4]. The temperature of storage represents one of the most important pre-analytical factors for the conservation of biological samples, as the use of an inadequate temperature can lead to degradation, oxidation and unfolding of the stored analytes over time and to consequently misleading results in e.g. biomarker discovery

experiments[47]. Furthermore, the storage time is also know to impact frozen samples, since even in carefully collected and stored samples long periods of time can alter levels of some of their components causing decreases or increases in marker concentrations[48,37,49]. The use of ultra-low temperatures (-80°C and -150°C) became standard for long-term storage to avoid biospecimens degradation[4]. Liquid nitrogen (LN_2) based storage is usually considered superior since it can reach temperatures below the glass transition temperature (Tg) of water (-137°C). It has been suggested that storage below this point may be optimal as degrading biochemical activities are thought to be inert[10], while degradation of proteins and RNA could still persist in samples stored at -80°C and was reported in different studies[50–52]

2.1.3.1 Cryopreservation

The increase of interest in cell therapies over recent years has focused attention on the processes that will enable product delivery in reliable, regulatory-compliant and robust ways[53]. One such consideration is cryobanking, which is required to sustain cell therapy delivery to the end user facilities at the required time, providing a quality assured product with necessary safety and potency characteristics[54,55]. Cryopreservation in its various forms is one of the main aid technology for cell therapies to be able to meet these demands. The common conception of cryopreservation is storage of living cells at the deep cryogenic temperatures provided by liquid nitrogen or the associated vapour phase (ranging from -196°C to approximately -170°C)[53]. A successful cryopreservation requires the development of protocols that achieves high cell recovery and maintain cell functionality, including proliferation potential, multi-lineage differentiation potential, immunomodulatory property, migration ability and secretory profile, which are identical to that of the cells at pre-storage state[54]. The slow freezing method is the preferred method of cryopreservation of high volume cells such as MSCs and cell lines, and with a freezing rate of 1°C/min, a large number of cells can be frozen at a low concentration of cryoproctective agents (CPAs)[4,56]. The roles of CPAs in cell cryopreservation are to stabilize the cell membrane, minimize osmotic stress to the cells, and protect cells against intracellular and extracellular ice crystal formation which are harmful to cells[57] (**Figure 2.2**). CPAs are divided into permeating CPAs (e.g., dimethyl sulfoxide (DMSO) and glycerol) and non-permeating CPAs (e.g., sucrose, trehalose, and foetal bovine serum (FBS)). Permeating CPAs can penetrate the cell membrane due to their low molecular weight and remove the water from the cells to prevent the formation of intracellular ice, while non-permeating CPAs are able to protect the cell membrane by forming a viscous glassy shell around the outer surface of the cells and regulate intracellular and extracellular osmotic pressure[58,59]. DMSO with a concentration of 10 % combined with FBS (20 – 90 %) has been commonly used to preserve human MSCs. Overall, an ideal CPA should maintain functional properties

and high survival rate of cells after thawing, and allow for cell transplantation without raising biosafety issues (e.g., xenogeneic immune response, cytotoxicity and tumorigenesis)[60].

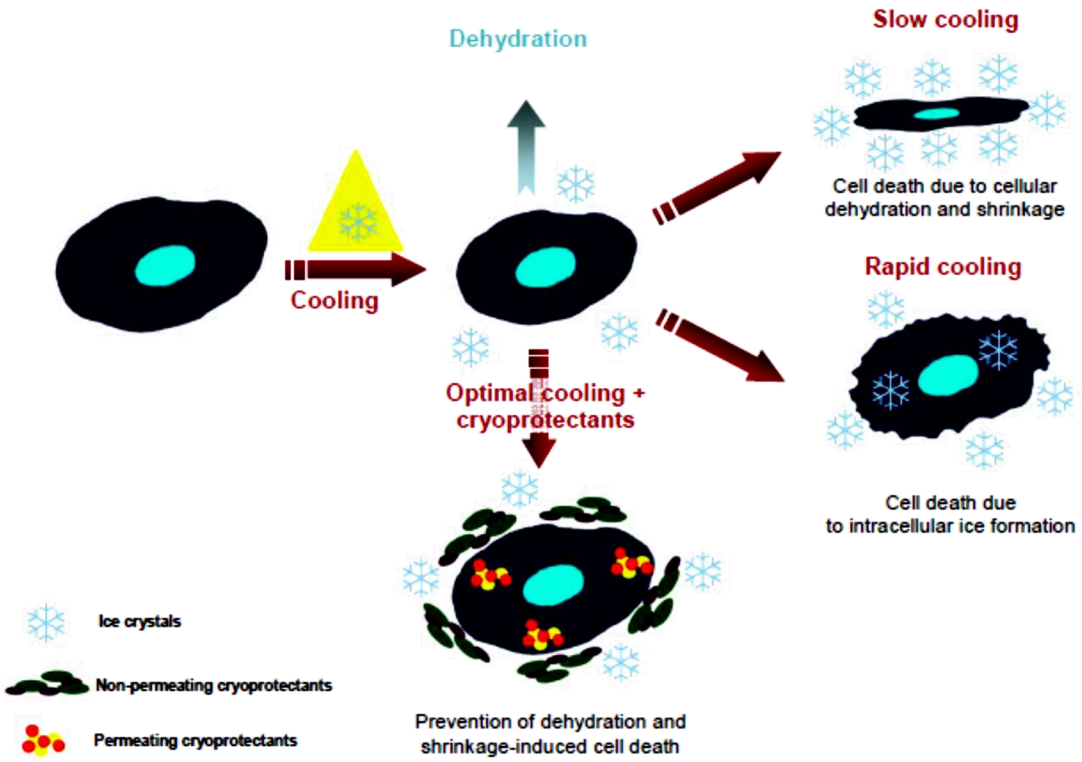

Figure 2.2 Effects occurring during the cryopreservation of cells at different cooling rates modified from Martín-Ibáñez et al[61]. When the cooling process starts, ice crystals formation is induced and free intracellular water is osmotically pulled from the cells. If the cooling process is slow, this effect leads to cell death by dehydration and shrinkage. In contrast, if the cooling process is rapid, intracellular ice crystals form before complete cellular dehydration has occurred. These crystals induce cell death by cellular organelles and membrane disruption during the thawing process. An optimal cooling rate together with the usage of cryoprotectants in the freezing media avoids dehydration effects and intracellular ice formation allowing cell survival after thawing.

2.1.3.1.1 GMP Compliance

CPAs like DMSO and FBS can however present some disadvantages. DMSO is cytotoxic at temperatures above 4°C[62]. Furthermore, the clinical uses of cells preserved in 10 % DMSO have caused many adverse effects (e.g., neurotoxicity and respiratory depression) in recipients[63,64]. Instead, FBS contains a number of proteins and peptides which can initiate xenogeneic immune responses and in addition, it could transfer pathogens[65,66]. For these reasons, Good Manufacturing Practice (GMP), a quality assurance system used by pharmaceutical manufacturers, is used in the production of stem cells for clinical application. GMP regulations, first promoted by the US Food and Drug Administration, and implemented in Europe as EU Directive 2003/94/EC[67], support the EU Tissue and Cells Directives and the Advanced Therapy Medicinal Products Regulations which concern the production of

haematopoietic, MSCs, hES and iPS cells for clinical therapy within most of Europe. The purpose of GMP is to ensure that these cell-based products are safe, pure and effective, by seeking to control all aspects of the production process from donor selection and harvesting/derivation of the cells, through enrichment/expansion to cell preservation, storage and distribution[55]. It requires traceability throughout the production chain and beyond (through systems for product recall and adverse event reporting) and is predicated on appropriate risk assessment and risk mitigation[68,69]. The major risks related to a cell based product are microbiological contamination, dedifferentiation/loss of cell function, cell transformation malignancies, immunogenicity and ectopic engraftment of cells to non-target tissues[70]. Definition and characterization of the product are of outmost importance, as these data provides the tools for proper process validation, in process testing and release testing. The product testing objectives are to demonstrate the proof of principle (specific therapeutic utilization) for the medicinal product and to define the pharmacological and toxicological effect that are predictive of the response in humans[53]. Further objectives comprise the establishment of safe doses for subsequent clinical studies and to support the route of administration of the cell-based product. Moreover, a non-clinical study also identifies target organs for toxicity and should allow for the definition of parameters to be monitored in the patients. It is also necessary to provide safety suitability and biocompatibility data for any additional substances that are administrated together or as a part of the cell-based medicinal product, such as cellular components, biomolecules, biomaterials and/or chemical substances[70].

2.2 Impact of Pre-analytics on Clinical Applications

In light of this, two important future clinical applications potentially biased by pre-analytics were chosen for this study. The main interests were on the applicability of storing liquid biopsy samples (serum) for pancreatic cancer screening and on the potential use of mesenchymal stromal (stem) cells for clinical applications, for example to heal bone fractures. Due to the unique setting at the Campus Lübeck with its centralized Hospital-integrated biobank infrastructure, it was possible to obtain, process and store serum samples and MSCs in a highly controlled fashion, and thus to analyse pre-analytical factors, such as sample processing methods, different storage temperatures, and duration of storage. These pre-analytical factors were chosen for their potential impact on the biomarker stability and quality of mesenchymal stem cell-based therapies. Pancreatic cancer was chosen as it remains one of the most lethal diseases with an increasing incidence in recent years[71]. Due to the inaccessible location of the pancreas as well as the lack of specific clinical markers[1], early detection of pancreatic cancer is still very difficult. MSCs were chosen as they have become quite popular in therapy applications over the years[72]. Their main application is in the repair of bone fracture, as

musculoskeletal diseases remain among the most prevalent and challenging clinical problems, especially for the elderly population[73].

2.2.1 Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer[74]. Other exocrine pancreatic neoplasms include acinar cell carcinoma, intraductal papillary mucinous tumour, mucinous cystic tumours, and serous cystic tumours. Endocrine pancreatic tumours represent about 5 % of cases and include islet cell and neuroendocrine carcinoma[75]. The pancreas is located in the retroperitoneum, where initial growth of the cancer remains silent; therefore, symptoms are usually a sign of advanced disease. Clinical presentation depends on the stage of disease and the location of the primary tumour: the pancreatic head, neck or uncinated process (70 %); the body or tail (20 %); or multifocal disease (10 %)[76]. The development of malignant pancreatic tumours from normal pancreatic tissue occurs through non-invasive precursor lesions known as pancreatic intraepithelial neoplasms (PanINs), intraductal papillary mucinous neoplasms (IPMN) or mucinous cystic neoplasms (MCN)[77]. Invasive pancreatic ductal cancer cells most commonly arise from PanINs while acquiring specific gene mutations as they progress from PanIN-1 to PanIN-3[78] (**Figure 2.3**). Pancreatic cancer grossly produces a firm, highly sclerotic mass. The edges of these cancers are poorly defined, with long tongues of carcinoma extending well beyond the main tumour[79]. The unique characteristics of PDAC microenvironment directly impact the molecular behaviour of cancer cells and it is considered to be the major risk factor for failure of immunotherapy and targeted therapies in patients[80]. It is known to be relatively dense and enriched by pancreatic stellate cells which produce a redundant amount of stromal elements including collagens, laminin, and fibronectin, and this is a process called desmoplasia[81,82].

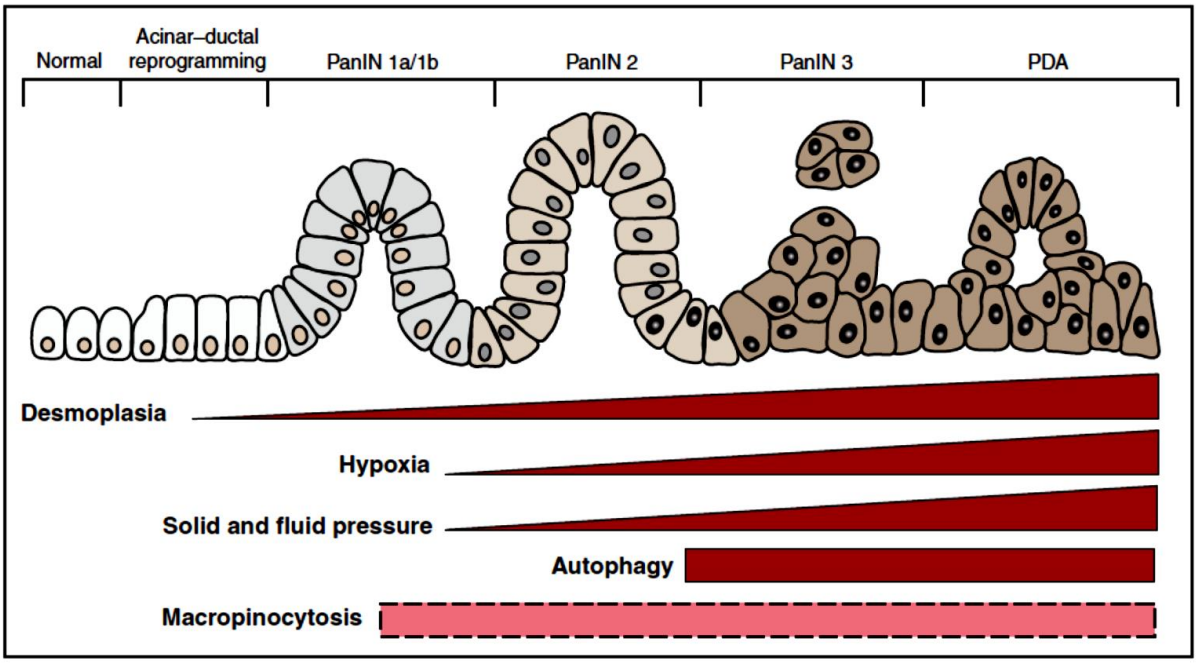

Figure 2.3 Schematic of the multistage progression of PDAC adapted from Perera and Bardeesy[81]. Invasive PDAC arises through multistage genetic and histologic progression from microscopic precursor lesions that are believed to develop and progress asymptomatically over several decades. PDAC has a unique microenvironment, which is characterized by a dense fibrotic stromal component (desmoplasia). An important consequence of the dense stroma is the generation of high levels of solid stress and fluid pressure in the tumours and compression of the vasculature, which creates a highly hypoxic and nutrient-poor microenvironment.

2.2.1.1 Epidemiology

2.2.1.1.1 Incidence and Mortality

Pancreatic cancer is one of the most lethal diseases, despite marked improvement in medical and cancer care over the past years, and its incidence increased significantly in recent years[71]. Although infrequent, it is the fourth leading cause of cancer death in the USA and leads to an estimated 227,000 deaths per year worldwide[83]. While the probability of developing PDAC is 30 % greater in males, age and racial/ethnic backgrounds play important roles in the incidence rates. The incidence rate for both sexes increases with age; pancreatic cancer is seldom diagnosed before 55 years of age, and it can be defined as a disease of elderly populations[84,85]. Of all diagnosed cases, about 27% are diagnosed between ages of 75 and 84, whereas about 9% are diagnosed between the ages of 45 and 54[76].

2.2.1.1.2 Prognosis

The 5-year survival rate for PDAC that is detected at an early stage is only 26 %. As PDAC progresses into the regional stage, in which the malignant cancer invades surrounding organs/tissues and the lymph nodes, the 5-year survival rate decreases to only 10 %. Detection is however more likely at the regional stage of the tumour (28 %)[76]. As with all cancers, once the primary tumour metastasizes to distant organs via the circulatory system, the probability of survival diminishes significantly. In the case

of PDAC, the 5-year survival rate for this disease is only 2 % once it has reached distant organs. Detection at this stage is 53 %, however, unfortunately only a few patients respond to the available interventions at this disease stage[86]. Despite the advancements in cancer research, the survival rate for PDAC did not improve significantly over the years. Between 1975 and 1977, the 5-year survival rate was 3 %. It increased to 4 % between 1987 and 1989 and currently, the 5-year survival rate is 7 %[86].

2.2.1.1.3 Aetiology and Risk Factors

Most cases of pancreatic cancer are sporadic with the major risk factors being aging, smoking, higher level of alcohol consumption, body fatness, adult-attained height, diabetes mellitus type 2, chronic pancreatitis and obesity[87,88]. It has been reported that overweight and obese individuals have an increased risk and earlier disease onset[89,90]. Current cigarette smokers and former smokers who had quit for less than 10 years also have a higher risk of pancreatic cancer than non-smokers[91,92]. Patients with diabetes are at greater risk for pancreatic cancer and new onset of diabetes may be an early indicator of pancreatic cancer[93,90]. Inherited genetic factors are also involved in the development of PDAC and are thought to contribute to 5 – 10 % of pancreatic cancers[94,95]. Moreover, a family history of pancreatic cancer is also an important risk factor for this disease, since individuals with a strong family history of pancreatic cancer have a significantly increased risk of developing the disease themselves[96,97]. Familial cancer syndromes like Peutz-Jeghers (PJ) syndrome, familial atypical multiple mole and melanoma syndrome (FAMMM), hereditary nonpolyposis colorectal cancer (HNPCC), or Lynch syndrome, familial adenomatous polyposis (FAP) syndrome, hereditary breast and ovarian cancer syndrome (HBOC) and hereditary pancreatitis (HP) also increase the risk of developing pancreatic cancer[71,98,99].

2.2.1.2 Histopathological Classification of Pancreatic Cancer

Once a diagnosis of PDAC is confirmed or highly suspected, an attempt to stage the tumour is made (**Table 2.1**). Unlike many other cancers, this is achieved primarily through triphasic computed tomography (CT) scan of the abdomen[100]. The criteria for defining resectable disease with CT favour specificity over sensitivity to avoid denying surgery to patients with a potentially resectable tumour[101]. Furthermore, the sensitivity of CT for small hepatic and peritoneal metastases is limited. In general, PDAC is considered resectable when there is no evidence of distant metastasis, there is a lack of tumour involvement of the major arteries, and in case of venous invasion it must be suitable for venous reconstruction[102]. If patients are considered borderline resectable, it is generally related to questionable involvement of the arteries and/or veins that cannot be fully assessed preoperatively.

Table 2.1 PDACs are classified regarding the local invasion depth (T stage), regional lymph node involvement (N stage), and presence of distant metastases (M stage). TNM staging system is according to the American Joint Committee on Cancer (AJCC) (8th edn.)[103] and the International Union for Cancer Control (UICC) classification is presented with resectability and 5-year overall survival rates for different TNM stages[104,105].

T stage – Primary Tumour	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary
Tis	*In situ*
T1	Limited to pancreas, ≤2 cm
T2	Limited to pancreas, >2 cm, ≤4 cm
T3	Maximum tumour diameter >4 cm
T4	Tumor involves the celiac axis, common hepatic artery or the superior mesenteric artery
N Stage – Regional Lymph Nodes	
Nx	Regional lymph nodes cannot be assessed
N0	No nodal metastasis
N1	Metastasis in 1–3 regional lymph nodes
N2	Metastasis in ≥ 4 regional lymph nodes
M Stage – Distant Metastasis	
Mx	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

UICC-Stage		**Resectable**	**5-year survival %**
Stage 0	Tis N0 M0	carcinoma in situ, resectable	n.a.
Stage IA	T1 N0 M0	localized, resectable	31 – 39 %
Stage IB	T2 N0 M0	localized, resectable	22 – 27 %
Stage IIA	T3 N0 M0	locally invasive, resectable, eventually borderline resectable when extended T3	16 – 25 %
Stage IIB	T1 N1 M0 T2 N1 M0 T3 N1 M0	locally invasive, resectable, eventually borderline resectable when extended T3	8 – 10 %
Stage III	T1 N2 M0 T2 N2 M0 T3 N2 M0 T4 Any N M0	locally advanced, borderline resectable or unresectable	0 – 7 %
Stage IV	Any T Any N M1	distant metastasis, palliative	0 – 4 %

2.2.1.2.1 Grading: Histomorphological Differentiation

Histopathological grading of PDAC is an important prognostic factor[106] and is performed according to the World Health Organization (WHO) defined histologic tumour grading system, including the presence of tubular structures vs. solid growth, the presence of mucin, nuclear polymorphism and number of mitoses[107]. Most pancreatic ductal adenocarcinomas are moderately to poorly differentiated, with varying degrees of duct-like structures and mucin production. Histologic grading,

which is based on the degree of differentiation and the prevalence of mitotic cells, typically uses three grade levels (grade 1, well differentiated; grade 2, moderately differentiated; grade 3, poorly differentiated), although highly anaplastic tumours are sometimes designated as grade 4[107].

2.2.1.2.2 Staging: Anatomic Extent of Malignancy

Pancreatic tumours are classified according to the local invasion depth of the primary tumour (T stage), involvement of regional lymph nodes (N stage), and presence of metastases in distant organs or lymph nodes (M stage). Depending on the imaging findings, patients with PDAC are generally divided into four groups: (1) resectable, (2) borderline resectable, (3) locally advanced (unresectable) and (4) metastatic[105]. Several staging systems or consensus statements developed by different societies or academic practices in the United States are used in clinical practice to stage patients with PDAC. These include those developed by the American Joint Committee on Cancer (AJCC)[103,108], the National Comprehensive Cancer Network[109], American Hepato-Pancreato-Biliary Association, Society of Surgical Oncology, the Society for Surgery of the Alimentary Tract and the University of Texas MD Anderson Cancer Centre[110] and the International Union for Cancer Control (UICC)[111]. These staging systems primarily depend on the tumour location, extension beyond the confinement of the pancreas, adjacent vessels contact or encasement/occlusion and the presence of distant metastatic disease. All of these staging systems are used to stratify patient outcome or suggest best treatment approaches depending on the stage of the disease[112]. Although the staging group or treatment suggestion for each patient may differ between the different systems, all do share common elements pertaining to tumour staging. These elements include the tumour size, location within the pancreas, extension beyond the pancreas with or without contact with the adjacent vessels, and the presence or absence of metastatic lesions[112].

2.2.1.3 Screening for Early Detection of Pancreatic Cancer

The best chance to cure this type of cancer is early detection combined with a radical surgical approach, which can lead to a 5-year survival rate of approximately 20 %, when combined with adjuvant chemotherapy. However, early detection is not so easy to obtain, due to the inaccessible location of the pancreas. Furthermore, this type of cancer is at the beginning asymptomatic and there is a lack of specific clinical markers for the early stages of pancreatic cancer[1]. Therefore, only 15 – 20 % of the cancer are diagnosed as resectable, while most of the patients are diagnosed as unresectable and chemotherapy is the standard treatment[3]. Hence, there is an urgent need to detect small asymptomatic cancers or precursor lesions, which are potentially curable[113]. The International Cancer of the Pancreas Screening (CAPS) Consortium proposed to define ‘‘successful screening” by detection and treatment of T1N0M0 margin negative pancreatic cancer and high grade dysplastic precursor lesions, including PanIN-3, IPMN with high grade dysplasia, and MCN with high grade dysplasia[114].

However, there is no ideal single screening method or program for detection of early pancreatic cancer yet. The most common screening imaging used for the detection of pancreatic cancer are endoscopic ultrasonography (EUS), computed tomography (CT) and magnetic resonance imaging (MRI) with magnetic resonance cholangiopancreatography (MRCP). However, these methods are either invasive or have low sensitivity[71]. An overabundance of biomarker-focused studies have been conducted to enhance the diagnosis and treatment of PDAC, nonetheless to little gain to date. The most widely utilized tumour marker for pancreatic cancer in the clinic is the carbohydrate antigen CA19-9[24]. It is beneficial in its analytical ability to monitor and detect post-treatment recurrence and its sensitivity in patients with symptoms suspected to be due to pancreatic cancer is approximately 80 %[115]. There are some limitations though, including elevated levels in benign disease, lack of expression in 5 – 10 % of the population (lacking the Lewis antigen glycosyl-transferase) and its restrictions to diagnose small/early tumours[116]. Studies have all shown poor results when exploring the utility of CA19-9 as a screening marker in early and asymptomatic cases with pre-invasive lesions[117]. Thus, the development of tumour markers that can replace serum CA19-9 in the diagnosis of asymptomatic pancreatic neoplasms, and particularly high-grade non-invasive lesions, is a priority. Promising candidates have been macrophage inhibitory cytokine 1 (MIC1), osteopontin (OPN), tissue inhibitor of metalloproteinase-1 (TIMP-1) and mesothelin (MSLN): studies in cohorts of pancreatic cancer and control patients have confirmed the potential utility of these markers in the diagnosis of early stage (i.e., resectable) pancreatic cancers[118–120]. Furthermore, according to the study of Meng et al, elevated serum carcinoembryonic antigen (CEA) levels could also play an important role in the clinical diagnosis of pancreatic cancer patients and predict poor prognosis, making CEA a vital supplementary to CA19-9[121].

2.2.2 Mesenchymal Stromal Cells: Definition and History

Mesenchymal stromal cells (MSCs) have emerged as prominent candidate for cell-based therapies, tissue repair and immune modulation[122]. MSCs are primitive cells originating from the mesodermal germ layer and were classically described to give rise to connective tissues, skeletal muscle cells, and cells of the vascular system[123]. They are spindle shaped plastic-adherent cells which can be isolated from bone marrow and present multipotent differentiation capacity in vitro[124]. These cells are capable of differentiation to give rise to bone (osteocytes), cartilage (chondrocytes) and fat (adipocytes) when induced in vitro[68]. In addition to bone marrow, MSCs were reported in an increasing range of tissues including peripheral blood, umbilical cord blood, placenta, amniotic fluid and membrane, dental pulp, deciduous teeth, adipose tissue, articular cartilage, brain, endometrium and menstrual blood and skin[122]. The incidence of these cells in tissue is extremely low, ranging from 0.00003% of nucleated cells in cord blood to 0.001-0.01% of nucleated cells in marrow, although this decreased with age[68]. It has

been widely accepted that primary MSCs cultures are a heterogeneous population of cells with varying capacities of self-renewal and differentiation[122]. MSCs were first observed by Friedenstein and colleagues in cultures of murine bone marrow. These cells after a few days enlarged into polygonal cells with characteristic features of osteoblasts or bone-forming cells. Thus, it became clear that aside from the blood-forming (hematopoietic) stem cells, non-hematopoietic cells exist in the bone marrow. Therefore, they described MSCs as hematopoietic supportive cells of bone marrow. Moreover, they proved that MSCs could differentiate to bone in vitro and a subset of these cells had a high proliferative potential when plated at low density in tissue culture and thus were capable of generating colony-forming unit-fibroblasts (CFU-F)[125,126]. Based on the work of Friedenstein, Owen proposed the existence of a stromal stem cell to maintain the marrow microenvironment[127]. The term of mesenchymal stem cells was first introduced by Arnold Caplan to designate cells in the mesoderm (middle germ layer), proposing that MSCs gave rise to bone, cartilage, tendon, ligament, marrow stroma, adipocytes, dermis, muscle and connective tissue[128] (**Figure 2.4**). MSCs lack a unique identifying phenotypic marker, therefore the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed a set of minimum criteria to define human MSC for both laboratory-based scientific investigations and for pre-clinical studies. MSCs must be plastic-adherent when maintained in standard culture conditions using tissue culture flasks, ≥ 95 % of the MSC population must express CD (cluster of designation)105, CD73 and CD90, as measured by flow cytometry. Additionally, these cells must lack expression (≤ 2 % positive) of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II. Furthermore, these cells must be able to differentiate to osteoblasts, adipocytes and chondroblasts under standard in vitro differentiating conditions[129]. Other surface markers generally expressed by MSCs include CD13, CD29, CD44, CD49a-f, CD51, CD10, CD105, CD106, CD166 and Stro-1[130].

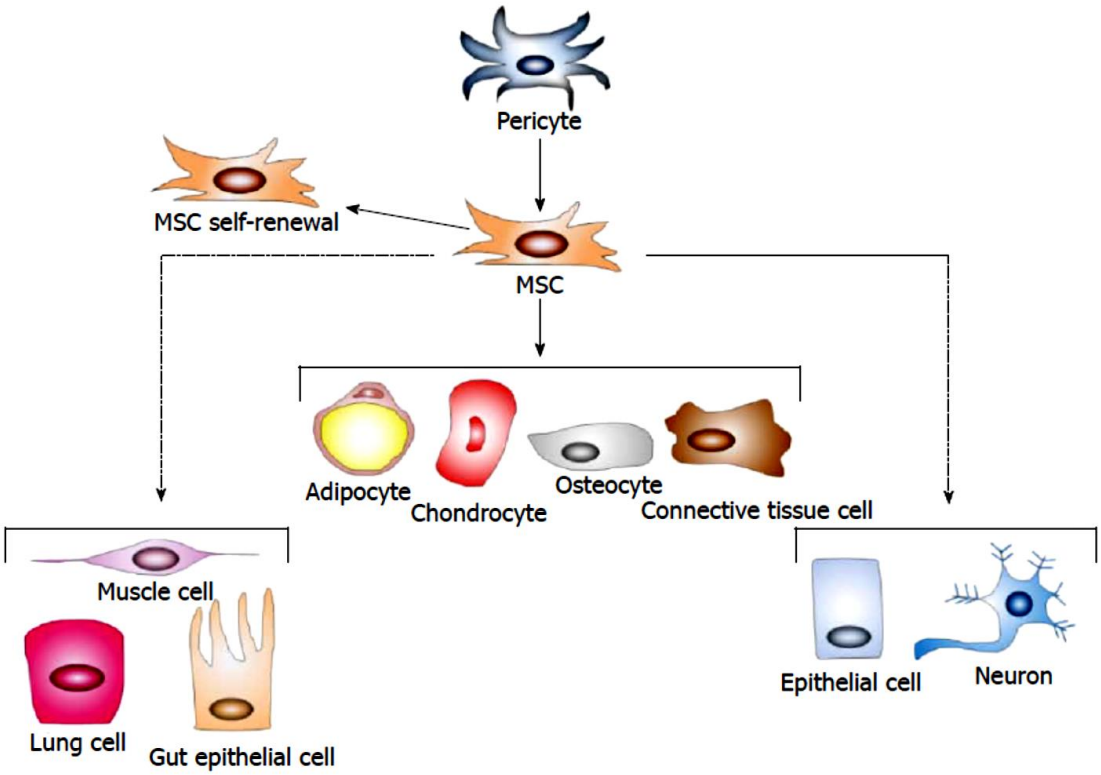

Figure 2.4 Basic properties of mesenchymal stem cells modified from Glenn et al[131]. MSCs are a heterogeneous population of stromal cells thought to be derived from pericytes. These cells are defined by self-renewal and the ability to differentiate into the mesodermal cells: adipocytes, chondrocytes, osteocytes, and connective tissue cells. Furthermore, though controversial, they may also transdifferentiate into cells of the endoderm (lung, muscle, and gut epithelial cells) and of the ectoderm (neurons and epithelial cells).

2.2.2.1 Controversies of MSCs Definition

The definition of "mesenchymal stem cells" was first introduced by Caplan in 1991[128]. However, this terminology has been criticized and considered technically inappropriate, as convincing data to support the "stemness" of these cells were not forthcoming. Most investigators recognized that in vitro isolated MSCs are not a homogeneous population of stem cells, although an authentic mesenchymal stem cell may reside within the adherent cell compartment[132]. Despite the attempts to establish the identity of MSCs, confounding issues remain such as the similarities that MSCs share with stromal fibroblasts[133] and with pericytes[134]. MSCs and fibroblasts exhibit a similar spindle-like morphology. In addition to this, both types of cells adhere to plastic and express the same surface markers[135]. Osteoblastic, chondrogenic, and adipogenic differentiation from fibroblasts has also been described[136]. Thus, data to support the designation of MSCs as biologically functional stem cells are lacking. However, the acronym "MSC" is deeply rooted in the language of cell biologists and clinical cell therapists. Thus, the ISCT has recommended that these spindle-shaped, plastic-adherent cells be termed, "mesenchymal stromal cells", allowing investigators to continue to use this acronym, which should reduce the potential for confusion in the literature[132].

2.2.2.2 Challenges

2.2.2.2.1 MSCs Heterogeneity

An important issue and challenge regarding MSCs is their heterogeneity. Tissue-dependent variation in differentiation capacity, surface markers and transcriptional and proteomic profiles is widely studied[137–139]. However, even when derived from the same tissue of origin, MSCs demonstrate huge donor-to-donor variability. Donor health may influence the availability and functional potential of MSCs[140,141] as MSCs availability, self-renewal capacity and differentiation potential have been reported to decline in older subjects[142–144]. However, even MSCs isolated from young, healthy donors exhibit strong differences in their proliferation rate, differentiation capacity, and ultimate clinical utility[145]. This functional variation extends to the molecular status of these cells. In the study of Mindaye et al, mass spectroscopy of MSCs isolated from six donors revealed that only 62 % of all identified proteins were found in at least half of the donors, and only 13 % of identified proteins were found in cells from each donor[146,147]. Furthermore, even in the same subject it is possible to find cell-to-cell variation amongst MSCs within a single population. In the study of Phinney et al., multiple bone marrow aspirates isolated from the same donor over a period of six months, or bilaterally from a donor at a single time point, yielded MSC cultures that proliferated at different rates[145]. Even within a single isolate, cell-to-cell variation in MSC phenotype becomes evident. Within subpopulations, further variation emerges during culture expansion, and is observable also in the functional differentiation capacity, molecular signature and the mechanical state of the cell[148]. A hypothesis is that these cells maintain a memory of their niche of origin, and this has an impact of their properties. In vivo, MSCs reside in niches characterised by broad cellular communities that present variable chemical and mechanical conditions. Indeed, micro-anatomical heterogeneity within the bone marrow niche has been shown to dictate cell-to-cell variation in osteolineage cells[149]. Therefore, upon isolation, it is not possible to keep MSCs from these heterogeneous environments separated, and existing in vivo variation may persist into in vitro cultures. There is mounting evidence that cultured cells retain a "memory" of their previous environments[150]. Furthermore, the mechanical properties of the stem cell microenvironment influence self-renewal capacity and regenerative potential[151]. Perhaps then, the mechanisms responsible for in vitro cellular memory may also facilitate the maintenance of heterogeneity in primary cultures[152].

2.2.2.2.2 Lack of Specific Surface Markers

A particular challenge to the field has been the absence of the unique set of markers that can be used to enrich MSCs from other connective tissue cell populations and define them functionally[153]. As mentioned before (cf. 2.2.2), fibroblasts express the same surface markers as MSCs and share also

other characteristics as plastic-adherence and the spindle-like morphology, thus the current definition suggested by the ISCT is incapable of separating them from MSCs[154]. For this reason, more studies are concentrating in finding new markers in order to determine which could be used for quick dividing of MSCs from fibroblasts[135]. Results from recent studies have suggested that the use of markers like CD10, CD26, CD106, CD146 and ITGA11 could be helpful for the discrimination of human bone marrow MSCs from human dermal fibroblasts[155–157]. However, knowing the high heterogeneity among MSCs populations, there is a need to confirm these surface markers by investigating their expression on MSCs and fibroblasts isolated also from other human tissues. Eventually, such markers could be used for the quality control of MSC cultures after expansion, cryopreservation, gene transfection and other manipulations[135].

2.2.2.2.3 Genomic Instability

Genomic stability and potential transformation leading to tumour growth has become one of the most important safety concerns for stem cell therapies[158]. High quantities of MSCs are needed for clinical applications, thus requiring extensive cell expansion in long-term culture, which increases the probability that genetic changes may arise[159]. Chromosomal alterations are associated with increased tumourigenicity and the inability to reach desired differentiation states. Some possible explanation for this kind of behaviour are MSC senescence[160,161] or tumour transformation[162]. Both phenomena could be explained by either contamination of adherent fraction of bone marrow (BM)-derived mononuclear cells (MNCs) with mature cells including fibroblasts or by adherent cells acquiring fibroblast-like phenotype over time[163,164]. Contaminating fibroblasts are known to undergo senescence and apoptosis in culture; whereas surviving cells become immortal, and if expanded further can become tumorigenic[165]. However, the occurrence of karyotypic instability in cultured MSCs has been documented. It has been admitted that genome instability enables tumour cells to acquire their characteristics[165]. Though, MSC studies presented highly conflicting results. The majority of the current literature suggests that MSCs have stable karyotypes[166–169], however it has also been shown that hBM-MSCs in vitro acquire chromosomal aberrations, undergo spontaneous transformation and form tumours in vivo[170,171]. Furthermore, questions remain about the contribution of individual donors to MSC genetic stability, what role the manufacturing process has on cytogenetic abnormalities, and the effect these abnormalities may have on the ability of these cells to perform their intended function while remaining safe[172]. Karyotypic abnormalities were found and can persist clonally, suggesting that monitoring genetic stability for this cell type should be part of the characterization of lines intended for clinical use[158].

2.2.2.3 MSCs Applications

Mesenchymal stromal stem cells have become quite popular for cell therapy applications, in particular since the limitation for the use of pluripotent embryonic stem cells (ESCs) in the clinic because of ethical considerations and the risk of teratoma formation after transplantation[173]. MSCs have a homing ability, meaning that they can migrate into injured sites, and they possess the capacity to differentiate into local components of injured sites and the ability to secrete chemokines, cytokines, and growth factors that can "sense" the requirements of the environment. These products can promote angiogenesis, regeneration, remodelling, immune cell activation or suppression, and cellular recruitment. At the same time the MSCs can also actively participate in bactericidal activity[174,175]. With the advancement of preclinical studies, MSCs have been shown to be effective in the treatment of many diseases, including cardiovascular diseases (e.g., myocardial ischemia and stroke), musculoskeletal diseases (e.g., osteoarthritis and rheumatic diseases), neurological diseases (e.g., spinal cord injury and amyotrophic lateral sclerosis) and immune system diseases (e.g., graft-versus-host disease (GvHD) and multiple sclerosis)[60,176]. MSCs possess some advantages for cell therapies compared to other cell types, like ESCs and induced pluripotent stem cells (iPSCs). They are easily available and easy to harvest, since MSCs can be isolated and expanded from the stroma of virtually all organs (cf. 2.2.1). Furthermore, they have multilineage differentiation potential, potent immunosuppressive effects and, as mentioned above, they lack ethical issues that occur with the application of human ESCs[177] (**Figure 2.5**). However, it is important to keep in mind that the trafficking of MSCs from their niche to target tissues is a complex process and may present some challenges, like the variability in outcome and the strength of effectiveness. One source of variability depends on whether the cells were derived from an allogeneic (different person) or autologous sources (self-derived)[175]. Furthermore, every MSC-donor is different (genetically, physiologically, etc.) and MSCs from different donors or sources are highly heterogenic; this clearly is one of the most important of the uncontrolled aspects of both allo- and autologous MSC-based therapies[175]. Other factors that influence the cell phenotype and their proliferation and differentiation potential, are the cell passages and culture conditions *in vitro,* cellular senescence and aging[178–180]. The known limited lifespan of MSCs in culture can guarantee safeness from malignancy, however it can also alter various cell functions including proliferation, differentiation and migration abilities that can limit the clinical usage. To expand the lifespan of hMSCs and maximize their clinical usefulness by improving their performance, various trials are under investigation[177].

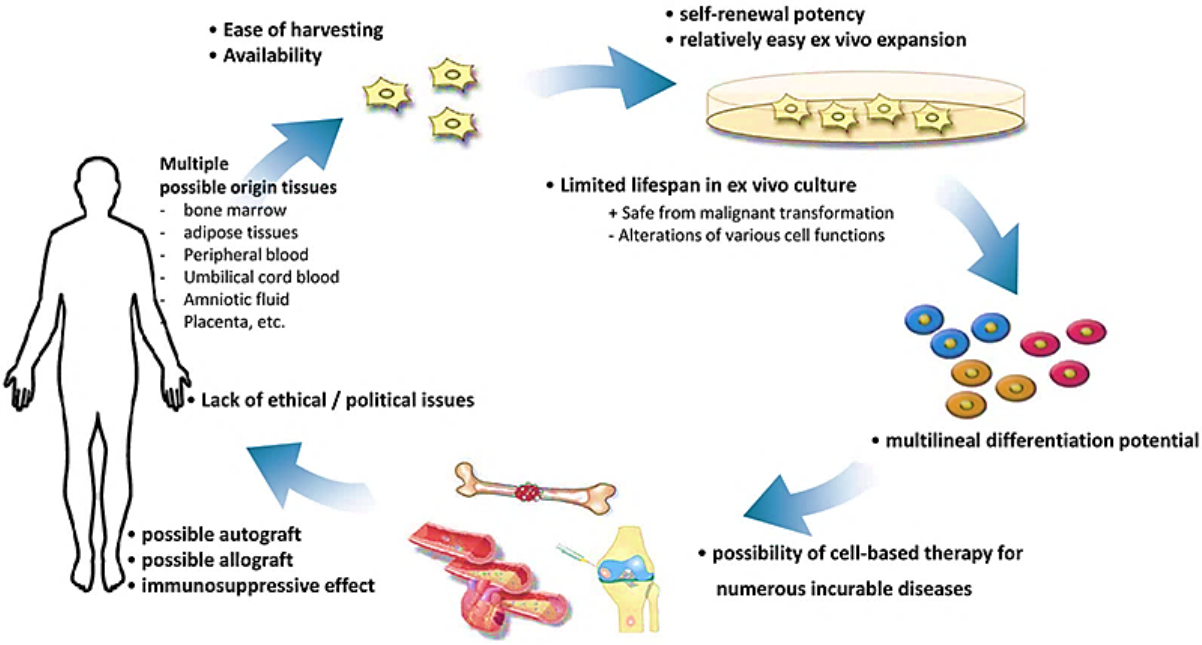

Figure 2.5 Advantages of human MSCs in cell-based therapy modified from Kim and Park[177]. hMSCs have several advantages for clinical use, such as the availability and ease of harvesting, multilineal differentiation potential, potent immunosuppressive effects, safety without any possibility of malignant transformation after infusion of allogeneic cells which is common in the case of ESCs and iPSCs, and the lack of ethical issues that occur with the application of human ESCs.

2.2.2.3.1 MSCs in Bone Fracture

Musculoskeletal diseases remain among the most prevalent and challenging clinical problems, especially for the elderly population. Although simple fractures often heal effectively, the fracture healing process is impaired in 10 – 20 % of cases, causing non-union and severe disability[73]. Furthermore, some fractures, such as hip fractures, are threatening injuries with mortality rates of 15 – 25 %[181]. Bone healing is a complex process of overlapping phases, including inflammation, repair, and remodelling that involves many intracellular signalling pathways which are responsible for regeneration of the new bone with the help of surrounding tissues. Bone has a self-repair ability so that it can repair itself when the nature and extent of injury is not large, chronic, severe, and complicated[182]. Nevertheless, in pathological fractures or large and massive bone defects, bone healing and repair fail. Insufficient blood supply, infection of the bone or the surrounding tissues, and systemic diseases can negatively influence bone healing, resulting in delayed unions or non-unions[183,73]. Moreover, there is a dynamic homeostatic interplay between bone formation and bone resorption, therefore an imbalance of bone remodelling such as bone formation not being able to compensate for ongoing bone resorption, is one of the main mechanisms leading to many bone diseases, such as osteoporosis and non-union of bone fractures[184]. MSCs have been shown to enhance bone regeneration in several preclinical and clinical studies by differentiating directly into bone forming cells and modulating the biological environment by secreting growth factors and anti-inflammatory

cytokines[185,186]. An adequate supply of cells (MSCs and osteoprogenitors) is important for efficient bone regeneration. The approach of delivering osteogenic cells directly to the regeneration site includes use of bone-marrow aspirate from the iliac crest, which also contains growth factors. It is a minimally invasive procedure to enhance bone repair, and produces satisfactory results[187,188]. However, the concentration and quality of MSCs may vary significantly, depending on the individual (especially in older people), the aspiration sites and techniques used and whether further concentration of the bone marrow has been performed, as bone-marrow aspiration concentrate (BMAC) is considered to be an effective product to augment bone grafting and support bone regeneration[142,188,189]. However, there are also significant ongoing issues with quality control with respect to delivering the requisite number of MSCs/osteoprogenitors to achieve adequate repair responses. Issues of quantity and alternative sources of MSCs are being extensively investigated. Novel approaches in terms of cell harvesting, in vitro expansion and subsequent implantation are promising, since in vitro expansion can generate a large number of progenitor cells[190].

3 Research Questions & Objectives

This study focused on the impact of pre-analytical conditions for biomarker research studies and the preservation of cell samples according to quality criteria. The main interest was to analyse different pre-analytical factors such as sampling, temperature and time of storage of serum samples and mesenchymal stromal (stem) cells (MSCs), which were stored in the local healthcare-integrated biobank. It is known that if samples are not stored correctly, degradation of their quality may occur, and this could lead, in the worst-case scenario, to a wrong diagnosis of the patient. Validated quality parameters and biomarkers could be useful tools for the evaluation of the optimum processing of any biosample, and, when used routinely, should allow for proper inclusion or exclusion of a sample or results from that sample. Therefore, the aims of this study were:

I. To verify if different temperatures of storage could affect the concentration and stability of six biomarkers (M2 pyruvate kinase (M2PK), interleukin-18 (IL-18), complement component 3a (C3a desArg), protein 26 (CD26), macrophage colony-stimulating factor (M-CSF), and S100 calcium-binding protein A11 (S100A11)) in serum samples over a time period of 1 year. For this purpose, serum samples without any storage were used to mimic the daily routine situation in clinical chemistry laboratories and were used as reference for protein concentration analyses.

II. To verify if different temperatures of storage could affect biomarker detection in a clinical study. For this purpose, four serum markers (C3a desArg, CD26, M-CSF and S100A11), detected by biochip array technology, and three markers currently used in clinical routine (Cyfra, CA19-9 and CEA) were analysed. Serum pancreatic cancer and healthy control patient samples were frozen at either -80°C or below -130°C.

III. To verify if different sampling methods could affect the quality of MSCs. Three different sampling methods were compared: a) direct isolation and cultivation of MSCs; b) storage of whole bone fragments below -130°Cwith subsequent isolation and cultivation of MSCs and c) cultivation of MSCs after isolation and storage below -130°C. Furthermore, a quality score to categorize the quality of MSCs samples (based on CFU, Proliferation Assay, differentiation ability (osteogenic & adipopogenic) ability, FACS markers) was developed.

4 Materials & Methods

4.1 Materials

4.1.1 Chemicals & Consumables

1α,25-Dihydroxyvitamin D_3 (Vitamin D_3)	Sigma-Aldrich, USA
2-Propanol, anhydrous, 99.5% ($(CH_3)_2CHOH$)	Sigma-Aldrich, USA
3-Isobutyl-1-methylxanthine (IBMX)	Sigma-Aldrich, USA
4',6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich, USA
Alizarin Red S (AR-S)	Sigma-Aldrich, USA
Ammonium bicarbonate (NH_4HCO_3)	Fluka, Switzerland
β-Glycerophosphate, Disodium Salt, Pentahydrate	Merck KGaA, Germany
Bovine Serum Albumin (BSA)	Sigma-Aldrich, USA
CellTiter-Blue® Cell Viability Assay	Promega GmbH
Citric acid monohydrate ($C_6H_8O_7$ x H_2O)	Merck KGaA, Germany
Cetylpyridinium chloride (CPC)	Sigma-Aldrich, USA
Cover slip, 24x40 mm	Menzel GmbH, Germany
Crystal violet dye	Sigma-Aldrich, USA
Dexamethasone ($C_{22}H_{29}FO_5$)	Sigma-Aldrich, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, USA
DMEM, high glucose, GlutaMAX$^{(TM)}$, pyruvate	Gibco® by Life Technologies, USA
Dulbecco's phosphate-buffered saline (DPBS)	Gibco® by Life Technologies, USA
Ethanol (C_2H_5OH), absolute	J.T.Baker, USA
Ethanol (C_2H_5OH), 99 %, complete denaturated	Th. Geyer GmbH, Germany
Foetal Bovine Serum (FBS)	Gibco® by Life Technologies, USA
Formaldehyde 37 %	Carl Roth, Germany
GlutaMAX™ Supplement	Gibco® by Life Technologies, USA
Horse Serum	Sigma-Aldrich, USA
Hydrochloric acid (HCl), 37 % p.a.	Merck KGaA, Germany
Insulin solution human	Sigma-Aldrich, USA
L-ascorbic acid phosphate magnesium salt n-hydrate (Vitamin C)	Wako Chemicals USA, Inc.

Lymphoprep™	StemCell Technologies
Magnesium chloride ($MgCl_2$)	Merck KGaA, Germany
MEM, Minimum Essential Medium	Gibco® by Life Technologies, USA
MEM Non-Essential Amino Acids Solution (100X)	Gibco® by Life Technologies, USA
Oil Red O	Sigma-Aldrich, USA
Paraformaldehyde (PFA)	Merck KGaA, Germany
Penicillin-Streptomycin (p/s)	Gibco® by Life Technologies, USA
Phosphate-buffered saline (PBS), pH 7.4, 1x	Gibco® by Life Technologies, USA
p-Nitrophenyl Phosphate, Disodium Salt, Hexahydrate	Sigma-Aldrich, USA
Rosiglitazone (BRL)	Cayman Chemical
Schiff's reagent	Sigma-Aldrich, USA
Sodium hydroxid (NaOH), 45 %	Carl Roth, Germany
Sodium bicarbonate ($NaHCO_3$)	Merck KGaA, Germany
Sodium chloride (NaCl)	Merck KGaA, Germany
Sodium Pyruvate (100 mM)	Gibco® by Life Technologies, USA
Trypan Blue solution	Sigma-Aldrich, USA
Trypsin-EDTA (0.05%)	Gibco® by Life Technologies, USA
Trizma® base ($NH_2C(CH_2OH)_3$)	Sigma-Aldrich, USA
Xylene (C_8H_{10}), ≥ 96 % p.a.	J.T.Baker, USA

4.2 Chemicals Stocks Preparation for Cell Culture

4.2.1 Osteogenic Differentiation

Vitamin C (50 mg/ml)	Vitamin C: 176.14 g/mol H_2O
Vitamin D_3 (10 µM)	Vitamin D_3: 416.64 g/mol Isopropanol (99.5 %)
β-Glycerophosphate (1 M)	β-Glycerophosphate: 216.036 g/mol H_2O
Dexamethasone (10 µM)	Dexamethasone: 392.46 g/mol
Stock 1:	Ethanol (99 %) = 0.001 M
Stock 2:	MEM Medium = 0.00001 M

4.2.2 Alkaline Phosphatase (ALP) Activity Assay

TBS (Trizma base 20 mM + NaCl 150 mM, pH 7.5)	Trizma base: 121.14 g/mol Nacl: 58.44 g/mol ddH_2O
3.7 % Formaldehyde – 90 % ethanol	Formaldehyde (37 %) Ethanol (99 %) H_2O
$NaHCO_3$ 50 mM, with 1 mM $MgCl_2$, pH 9.6	$NaHCO_3$: 84.01 g/mol $MgCl_2$: 203.03 g/mol ddH_2O
P-nitrophenylphosphate	1 mg/ml P-nitrophenylphosphate 50 mM $NaHCO_3$ + 1 mM $MgCl_2$
3N NaOH	5N NaOH ddH_2O

4.2.3 Alizarin Red Staining

70 % ethanol	Ethanol (99 %) ddH_2O
10 % CPC	Cetylpyridinium chloride: 339.992 g/mol H_2O

4.2.4 Adipogenic Differentiation

IBMX (10 mg/ml = 45 mM)	3-Isobutyl-1-methylxanthine: 222.3 g/mol Ethanol (99 %)
BRL (1 mM)	Rosiglitazone: 357.4 g/ml Dimethyl sulfoxide: 78,13 g/mol

4.2.5 Oil Red O Staining

3 % isopropanol	Isopropanol (99.5 %) H_2O
4 % paraformaldehyde	PFA PBS, pH 7.4, 1x

4.2.6 Flow Cytometry

FACS Buffer	BSA (1 %) PBS, pH 7.4, 1x

4.3 Clinical Materials for Serum Proteomics

4.3.1 Patient Sample Collections

Studies were approved by the local Ethics Committee (local ethical review board No #07-124, 07-194, 16-281, 16-282 and 19-147A) at the *University of Lübeck*. After signed consent, peripheral blood of healthy volunteers and patients were collected, processed, and stored at the *Interdisciplinary Centrum for Biobanking-Lübeck (ICB-L)*. At the time of diagnosis and prior to any therapy, clinical material was obtained from patients with diagnosed pancreas cancer at the University Hospital Schleswig-Holstein (UKSH), Campus Lübeck. Tumours classified as PDAC (pancreatic ductal adenocarcinoma) were pathologically staged according to the TNM classification and the system of UICC. Pancreas cancer patients were divided into an early-stage (UICC I & II) and a late-stage (UICC III & IV) group. Patients/volunteers with a negative colonoscopy and without any history of cancer were used as healthy controls.

4.3.2 Serum

Serum was obtained from clotted, venous blood samples by centrifugation at 2000 x g for 10 min. Until further use, sera were stored in the gas-phase of liquid nitrogen at a temperature below -130°C or in -80°C freezers.

4.3.2.1 Serum for Pre-analytical Stability of Biomarkers

Proteomic analysis for the stability cohort study derived from serum was performed using samples from five healthy volunteers from the *Section for Translational Surgical Oncology and Biobanking, Department of Surgery, University Hospital Schleswig Holstein, Campus Lübeck*. All serum samples were pooled together in order to reduce the number of variables (e.g., gender, age) that could influence the analysis and the comparison of methods. The serum was divided into different aliquots (**Figure 4.1**) in order to be analysed at different time-points: without storage (fresh serum), after 3 days, after 2 weeks, after 3 months and after 1 year. The aliquots were stored at different temperature conditions: room temperature (20°C), -80°C, below -130°C (N_2), and -80°C with subsequent movement to below -130°C after 1 hour to simulate gradient freezing (-80°C/N_2).

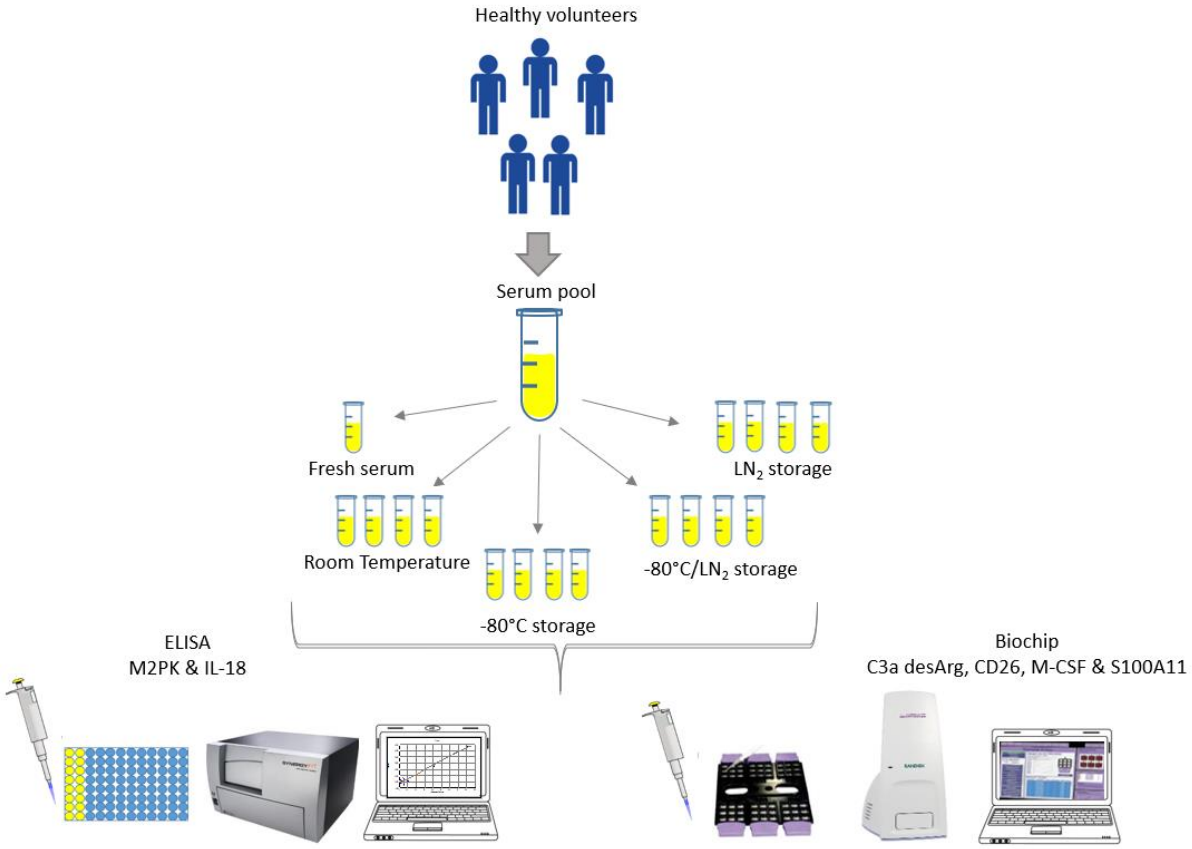

Figure 4.1 Schematic representation of the study design. The sera of five volunteers were pooled after centrifugation and divided into 17 aliquots: one aliquot for the fresh serum analysis, four aliquots were stored at room temperature, four aliquots at -80°C, four aliquots at <-130°C and four aliquots were stored for one hour at -80°C and moved subsequently to <-130°C. The samples were run in 10 replicates on ELISA and biochip technology. The experiment was repeated after three days, two weeks, three months and one year.

4.3.2.2 Serum for Pre-analytic Impact for Clinical Biomarker Research

Proteomic analysis for pancreatic cancer screening derived from serum was performed in a multicentre study in cooperation with the members from the *Division of Clinical Epidemiology and Aging Research at the German Cancer Research Centre in Heidelberg*, the *General, Visceral and Thoracic Surgery Department at the University Hospital Hamburg-Eppendorf*, the *Institute for Experimental Cancer Research at the Christian-Albrechts University in Kiel*, the *Molecular Biology Laboratory, Visceral, Thoracic and Vascular Surgery Department at the Philipps University in Marburg*, the *Institute for Laboratory Medicine at the German Heart Centre Munich and Technical University Munich*, and the *Molecular Oncology and Immunotherapy Laboratory, Department of Surgery, University of Rostock*. For this study serum samples were stored either at -80°C (serum samples from Kiel, Marburg, München and Heidelberg) or in the gas-phase of liquid nitrogen at the temperature of <-130°C (serum samples from Lübeck, Hamburg, Rostock) (**Table 4.1**). The samples were comprising healthy controls (n=66) and pancreatic adenocarcinoma (n=472). Detailed clinical data of the patient cohort are summarized in **Table 4.2** and **4.3**.

Table 4.1 Study cohort with the samples divided per collection site, temperature of storage and entities.

	Lübeck	**Hamburg**	**Rostock**	**Kiel**	**Heidelberg**	**Marburg**	**Munich**	**Total**
Storage temperature	*N_2*	*N_2*	*N_2*	*-80°C*	*-80°C*	*-80°C*	*-80°C*	
Healthy controls	37				24		5	**66**
Pancreas cancer	56	54	25	248	40	30	19	**472**
Total	**93**	**54**	**25**	**248**	**64**	**30**	**24**	**538**

Table 4.2 Summary of clinical data consisting of pancreatic cancer patients and healthy controls. The Heidelberg samples are not represented in this table, as no clinical data is available.

**for 3 pancreas cancer patients no information about the gender*

***for 1 pancreas cancer patient no information about the gender*

		-80°C & <-130°C		**<-130°C**	
Parameter	Value	Pancreas cancer patients (n=431)*	Healthy control patients (n=42)	Pancreas cancer patients (n=135)**	Healthy control patients (n=37)
Gender	Female	206 (47.8%)	21 (50%)	65 (48.1%)	18 (48.7%)
	Male	222 (51.5%)	21 (50%)	69 (51.1%)	19 (51.3%)
Age (years)	Range	38 – 87	33 – 84	38 – 84	33 – 84
	Mean	65.86	59.76	65.7	59.7

Table 4.3 Summary of clinical data of the pancreatic cancer patients. The Heidelberg samples are not represented in this table, as no clinical data is available.

		-80°C & <-130°C		**<-130°C**	
Parameter	Value	Pancreas cancer patients	%	Pancreas cancer patients	%
UICC stage	1	16	3.7	6	4.4
	2	71	16.4	19	14
	3	180	41.7	67	49.6
	4	84	19.5	13	9.6
T status (tumour size)	1	5	1.3	3	2.2
	2	20	4.6	9	6.6
	3	231	53.6	84	62.2
	4	46	10.6	6	4.4
N status (nodal status)	0	84	19.5	25	18.5
	1	199	46.1	74	54.8
	2	2	0.4	1	0.7
	3	2	0.4	1	0.7
M status (distant metastasis)	0	123	28.5	9	6.6
	1	79	18.3	9	6.6
Tumour grading	G1	37	8.6	9	6.6
	G2	189	43.8	54	40
	G3	97	22.5	32	23.7

4.4 Clinical Materials for Bone- and Cell-based Technologies

Studies were approved by the local Ethics Committee (local ethical review board No 16-085 and 16-086) at the University of Lübeck. After signed consent, bone tissue samples of patients were collected, processed and stored at the *ICB-L*. The bone tissue was collected from patients undergoing bone fracture surgery and comprised of “left-over” bone material, which would have otherwise been thrown away after operation.

4.4.1 Patient Samples Collection

The bone samples were collected after surgery and brought from the operation room to the cell culture laboratory, where they were divided into smaller pieces of the trabecular bone. Part of the bone was stored in pre-weighted 2 ml cryo-tubes (FluidX, Brooks Life Science Systems) with a standard cryopreservation medium (90% FBS + 10% DMSO) to be afterwards stored below -130°C for subsequent analysis, while the rest was directly processed in order to isolate mesenchymal stromal stem cells. Furthermore, a part of the cells isolated was resuspended with cryopreservation medium, collected into 2 ml cryo-tubes (1.8 ml cryopreservation medium per tube) and frozen with a gradient freezing method (Mr. Frosty) below -130°C for further analysis while the rest of the cells was seeded in culture. A total of 30 samples were collected from 25 patients comprising the following distinct material type: femoral heads (n=20), humeral heads (n=3), acetabulum (n=3), distal femur (n=2) and proximal tibia (n=2). Of these, 12 samples (femoral heads (n=8), humeral heads (n=2), acetabulum (n=2)) were used for the establishment of mesenchymal stromal stem cell isolation, culture and differentiation within the *Section for Translational Surgical Oncology & Biobanking, Department of Surgery, University of Lübeck and University Hospital Schleswig-Holstein (UKSH), Campus Lübeck*. For the present study, only femoral head samples (n=8) were used, in order not to include too many variables that could have influenced the analysis. Detailed clinical data of the patient cohort are summarized in **Table 4.4**.

Table 4.4 Summary of clinical data of the study group consisting of femoral head patients

Parameter	Value	Femoral head patients (n=8)
Gender	Female	5 (62.5 %)
	Male	3 (37.5 %)
Age (years)	Range	57 – 97
	Mean	71.2

4.5 Serum-based Methods for Protein Biomarker Discovery

In the following, serum-based methods are described in accordance with standard operation procedures (SOPs) of the *Section for Translational Surgical Oncology & Biobanking, Department of Surgery, University of Lübeck and University Hospital Schleswig-Holstein (UKSH), Campus Lübeck* and of *Randox Laboratories Ldt., Crumlin, UK.*

4.5.1 Multiplex Randox Biochip

The Evidence Investigator Biochip Array technology is used to perform a simultaneous quantitative detection of multiple analytes from a single patient sample. The core technology is the Randox Biochip, a solid-state device containing an array of discrete test regions of immobilized antibodies specific to different cancer markers. Two biochip platforms were designed for the detection of nine-biomarkers as biochip array in combination with the Evidence Investigator Analyser (Randox Laboratories Ldt., Crumlin, UK) and are based on simultaneous chemiluminescent sandwich immunoassays. Capture antibodies, specific for each biomarker are bound to the biochip surface defining arrays of test sites.

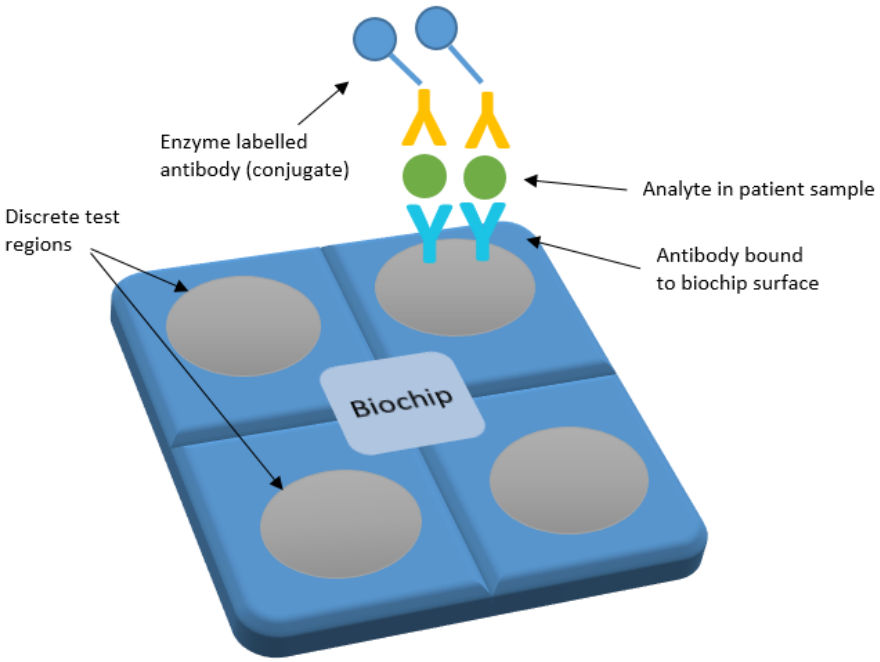

Figure 4.2 Sandwich immunoassay adapted from Randox Ltd (*https://www.randox.com/evidence-investigator/*). The analytes in the patient samples are bound by the capture antibodies, immobilized in the array of discrete test regions on the biochip.

4.5.1.1 Determination of Biomarker Serum Levels

The system allows handling of up to 54 samples (6 x 9 wells) in biochip carriers. Three controls and a nine-point calibration curve can run in parallel with 42 patient samples. The inclusion of controls and calibration samples avoids the need for technical replicates for patient samples. Upon completion of the immunoreactions, the chemiluminescent signal in each test site was detected with a super cooled charged coupled device (CCD) incorporated with the system data automatically processed. The analyser routinely assesses the quality of assay performance and generates calibration curves as described by FitzGerald et al[191]. The analytes' concentration present in the sample is calculated automatically using generated calibration curves (Evidence Investigator Software version 1.4). Due to different assay- and detection ranges of some biomarkers, the nine chosen serum markers had to be

subdivided onto two biochip platforms: One biochip comprised C3a desArg, CD26 and CRP, and the other chip incorporated IL-8, CEA, VEGF, M-CSF, S100A11 and NNMT. Both chips were manufactured according to standards described[191,192], and assay ranges and sensitivities for each analyte were measured (**Suppl. Table 9.1**).

4.5.1.2 Development of the Multiplex Biochips

Target analytes for the arrays were based on an extensive review of the literature[193] and own experimental data[194–196]. Design input requirements were determined following literature specifications. The product was manufactured and validated on 100 serum samples according to Randox Laboratories' manufacturing guidelines and procedures. The validation followed a series of approved standard operating procedures. The sensitivity, recovery and precision of each assay were determined. Sensitivity was evaluated to determine the lowest concentration that could be accurately detected for an assay. Precision was assessed both within runs (intra) and between runs (inter). Three samples of known concentrations which span the assay range were assessed 20 times as a measure of the intra-assay precision. The precision is expressed as the co-efficient of variation (%) over the 20 replicates. Inter-assay precision implies the assessment of these 3 samples in duplicate over 10 separate runs. Again, the precision was assessed as the co-efficient of variation (%) over 20 replicates. Deviation of ≤ 15% was deemed acceptable for both intra- and inter-assay precision.

4.5.1.3 Optimization of the Multiplex Biochips

A pilot study was conducted with the developed biochip arrays. The training set was composed of 1,033 samples of different entities: healthy controls (n=207), colon adenoma (n=142), colon cancer (n=274), rectal cancer (n=105), pancreas cancer (n=83), pancreas adenoma (n=15), diverticulitis (n=60), stomach cancer (n=15), lung cancer (n=50), liver cancer (n=10), breast cancer (n=20), ovarian cancer (n=4), liver metastasis (n=25), lung metastasis (n=13), prostate cancer (n=10). Detailed clinical data of the patient cohort are summarized in Suppl. Tables 9.2a and 9.2b. An independent quartile-based predictive model showed the best clinical performance for detecting pancreatic carcinoma, using a combination of M-CSF, S100A11, C3a desArg and CD26 (AUC = 0.77). The biochip was subsequently redesigned to compose four markers, which are divided onto two biochips according to their detection range. Thereby, one biochip comprises C3a desArg and CD26, and the other chip incorporates M-CSF and S100A11. The new assay ranges were: 0 – 12,000 ng/ml for C3a desArg, 0 – 4,000 ng/ml for CD26, 0 – 500 pg/ml for M-CSF and 0 – 50 ng/ml for S100A11. To exclude batch effects, the newly manufactured biochips were tested with a subset of samples of the previous study, which comprised healthy controls (n=168) and pancreatic cancer patients (n=74). The results were consistent with the first study, showing statistically significant results (AUC = 0.74) and confirming a good clinical

performance of the biochips for the detection of pancreatic carcinoma. Detailed clinical data of the patient subset cohort are summarized in **Suppl. Table 9.3a** and **9.3b**.

4.5.1.4 Multiplex Biochips Workflow

The samples were thawed on ice before the analysis, and all materials were equilibrated to room temperature prior to use. The calibrators and controls are constituted of 9 and 3 vials respectively (per array) of lyophilized base material containing analytes for the full array. These vials were reconstituted in 1 ml of deionized distilled water at 20°C to 25°C and placed on the shaker for 30 minutes out of bright light before use. 200 µl of assay diluent (20 mM Tris Buffered Saline pH 7.2 containing protein, surfactant, blocking agents and preservatives) (for biochip I) or 210 µl assay diluent (for biochip II) were pipetted per wells. Subsequently, 100 µl of calibrators and controls and 1:200 diluted serum sample (10 µl sample in 1990 µl sample diluent, for biochip I) or 90 µl undiluted serum (for biochip II) were pipetted per well. The samples were then incubated into a Thermoshaker (Randox Laboratories Ltd., Crumlin, UK) for 60 min at 37°C and 370 rpm. Following the incubation time, the reagents were discarded into waste using a sharp, flicking action of the handling tray and two quick wash cycles were performed using the diluted wash buffer (dilution according to manufacturer´s instructions). The first two quick washing steps were followed by six wash cycles, in which the biochips soaked in the wash buffer for 2 min before discarding the liquid. After the final wash, the carrier was gently tapped onto lint free tissue to remove any residual wash buffer and 300 µl of conjugate (20 mM Tris Buffered Saline pH 7.5 containing protein, surfactant, preservatives and assay specific antibodies labelled with horseradish peroxidase) were immediately pipetted into each well. The biochips were placed into the Thermoshaker for a second incubation of 60 min at 37°C and 370 rpm. Following incubation, the reagents were discarded into waste and again two quick wash cycles were performed, followed by six wash cycles with 2 min soak time each into the wash buffer. After the final wash, the wells were filled with wash buffer and left to soak until directly prior to imaging. The carriers were processed individually and those awaiting imaging were protected from light. The carrier to be imaged was filled with 250 µl of working signal reagent, composed of Luminol-EV840 and Peroxide in a 1:1 ratio, and it was covered from light and incubated for 2 min (+/- 10 sec). Finally, the carrier was placed inside the Evidence Investigator to acquire the imaging signal.

4.5.2 Enzyme-linked Immunosorbent Assay

The enzyme linked immunosorbent assay (ELISA) is a powerful method for detecting and quantifying a specific protein in a complex mixture. Originally described by Engvall and Perlmann[197], the method enables analysis of protein samples immobilized in microplate wells using specific antibodies. In a typical assay designed to detect an antigen in a complex protein mixture, the antigen is immobilized

either by direct adsorption or via an antibody adsorbed to the wells of a microplate. The plate is blocked and the antigen is probed with a specific detection antibody. The detection antibody may be directly labelled with a signal-generating enzyme or fluorophore or it may be secondarily probed with an enzyme- or fluor-labelled secondary antibody. For enzymatic detection, the appropriate enzyme substrate is added. The signal observed is proportional to the amount of antigen in the sample. Washing between steps ensures that only specific (high-affinity) binding events are maintained to cause signal at the final step. Sandwich ELISAs have become very popular when using complex protein samples since only the specific antigen becomes immobilized rather than the entire sample of proteins (**Figure 4.3**). The more antigen that is immobilized, the higher is the potential sensitivity of the assay. Sandwich ELISAs require two different antibodies that bind specifically to the antigen (each reacting with a different epitope). The first antibody (bound to the plate) is called capture antibody or coating antibody, whereas the second antibody detects the immobilized antigen and is called detection antibody. Such antibodies are known as "matched pairs"; they must be validated to work in combination, as they must not compete for binding to the antigen for accurate results to be possible.

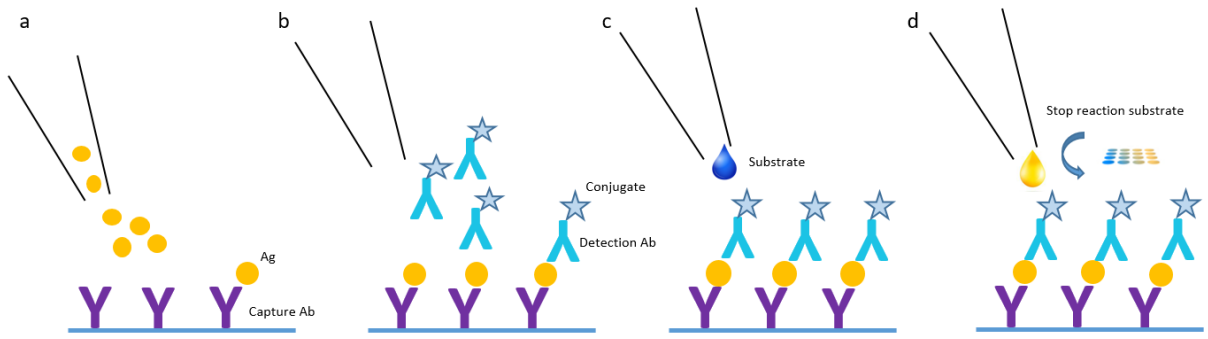

Figure 4.3 Sandwich ELISA immunoassay; a, the analytes are fixed to the capture antibody; b, the conjugate bound detection antibody binds with the analytes; c, substrate is pipetted, which gives a colour reaction after an incubation time that change the wells to a blue colour; d, the stop reaction substrate is added, changing the colour to yellow.

4.5.2.1 Randox M2PK & IL-18 ELISA

The ELISA kits provided by Randox (Randox Laboratories Ltd.) comprise a colorimetric sandwich immunoassay for the detection of M2PK and IL-18 in human serum samples. The detection of the proteins is performed through a catalytic reaction of the Horseradish Peroxidase (HRP) and a chromogenic substrate is used. These two ELISAs have been developed to eliminate interference by other components known to be present in biological samples. The measure ranges are 0 – 1,280 ng/ml for M2PK and 0 – 12,500 pg/ml for IL-18. The sensitivity, recovery and precision of each assay were determined. Sensitivity was evaluated to determine the lowest concentration that could be accurately detected for an assay. Precision was assessed both within runs (intra) and between runs (inter). Six

replicates of samples were analysed at each standard level to determine the intra-assay precision. The precision is expressed as the co-efficient of variation (%) over the six replicates. Inter-assay precision implies the assessment of two independent samples in duplicate on three separate days. Deviation of ≤ 20% is acceptable for both intra- and inter-assay precision. The sensitivity of the M2PK assay was determined to be 3.4 ng/ml, while the limit of sensitivity of the IL-18 assay was determined to be 42.8 pg/ml. Assay ranges and sensitivities for both analytes are shown in **Suppl. Table 9.4**.

4.5.2.2 Randox M2PK & IL-18 ELISA Workflow

The samples were thawed on ice before the analysis, and all reagents were equilibrated to room temperature prior to use. The stock solution of the standards was constituted of 1 vial (per array) of lyophilized base material and was reconstituted in 1ml of deionized distilled water at 20°C to 25°C and placed on the shaker for 30 minutes out of bright light before use. To prepare the standards, serial dilutions of the stock solution were performed. The microtiter plate was activated before use by three washing cycles with diluted wash buffer (dilution according to manufacturer´s instructions) with 1 min soak time after each wash. Samples were diluted 1:4 for this study. 100 µl of each standard were pipetted into the appropriate wells in duplicates, subsequently 100 µl of the diluted samples (25 µl sample in 75 µl in assay diluent) were pipetted into the appropriate wells and the microtiter plate was covered with adhesive plate sealer and incubated for 1 hour at 37°C in the dark. Following the incubation time, the reagents were discarded into waste using a sharp, flicking action of the plate and five wash cycles were performed, with one minute soak time in the wash buffer before discarding the liquid. After the final wash, the plate was gently tapped onto lint free tissue to remove any residual wash buffer and 100 µl of the Conjugate solution were immediately pipetted into each well. A second incubation of 1 hour at 37°C in the dark was performed. Following incubation, the reagents were discarded into waste and again five wash cycles were performed, with one minute soak time each into the wash buffer. Immediately after washing, 100 µl of the *One-Shot* Substrate were pipetted into the wells and the plate was incubated for 10 min at room temperature in the dark. The colour reaction was then stopped by the addition of 100 µl of Stop Solution per well and a colour change from blue to yellow was evident. The plate was then placed into the ELISA-Reader Synergy HAT (BioTek, Winooski, USA) as soon as possible after stopping the colour reaction and the optical density was measured at 450 nm. The software Gen5 (Erlab, Köln) was used to create a standard curve to determine the concentration of the markers in the samples.

4.6 Tissue- and Cell-based Methods for Mesenchymal Stromal Stem Cell Isolation, Culture and Differentiation

In the following, tissue- and cell-based methods are described in accordance with standard operation procedures (SOPs) of the *Section for Translational Surgical Oncology & Biobanking, Department of Surgery, University of Lübeck and University Hospital Schleswig-Holstein (UKSH), Campus Lübeck,* of the *Clinic for Orthopaedic and Trauma Surgery, University Hospital Schleswig-Holstein (UKSH), Campus Lübeck* and of the *Department of Endocrinology and Metabolism, University Hospital of Odense, Denmark.*

4.6.1 Density Gradient Centrifugation

First described by Böyum[198] and Koistinen[199], density gradient techniques are used since they are technically simple and highly reproducible. Lymphoprep™ is a density gradient medium recommended for the isolation of mononuclear cells (MNCs) from peripheral blood, cord blood, and bone marrow by exploiting differences in cell density. Granulocytes and erythrocytes have a higher density than mononuclear cells (MNCs) and therefore sediment through the Lymphoprep™ layer during centrifugation. The bone sample collected from the operating room was first divided into small pieces of trabecular bone, as this is considered a good source for mesenchymal stromal stem cells[200]. A part of the bone was stored in nitrogen, as mentioned above (cf. 1.4.1), while the rest of the trabecular bone was used for the isolation of the cells. The sample was transferred into a 50 ml Falcon tube and 20 ml of culture medium were added so that the bone was completely covered by the liquid. The sample was then vortexed for 30 seconds and afterwards the above part was transferred into a new Falcon tube. This step was repeated 4 times. Afterwards, 15 ml of Lymphoprep™ and 35 ml of the sample suspension obtained from the four vortex cycles were pipetted into new Falcon tubes. The samples were then centrifuged for 25 minutes at 2200 rpm at 19°C without brake. The ring of cells formed on the middle part of the tube was collected with caution and pipetted into another tube. The tube was filled with DPBS, to a total volume of 50 ml. The tubes were then centrifuged for 7 minutes at 1500 rpm at 4°C with brake. The supernatant was aspirated and the cell pellet was resuspended with 5 ml of culture medium. The number of cells was counted on the microscope with the Neubauer chamber (BLAUBRAND, Germany), using 10 µl Trypan blue and 10 µl of the cell suspension. The calculation was: (4 Squares/4) x 2 x 10,000. In case of too high cell number, more culture medium was added to the cell suspension and the calculation repeated. 3 x 10^6 cells were seeded for the Colony-Forming Unit Assay (CFU) in three Petri-dishes (1 x 10^6 cells/19.6cm^2), while 10 x 10^6 cells were seeded per T75 flasks. A part of the cells obtained from the density gradient centrifugation was not seeded, but was centrifuged for 5 min at 1880 rpm at 19°C. As mentioned above (cf. 1.4.1), the pellet was

resuspended with cryopreservation medium, collected into cryo-tubes and frozen with Mr. Frosty below -130°C.

4.6.2 Mesenchymal Stromal Stem Cell Culture

For the first three times, the medium was changed with a 1/3 ratio, in order to give time to the cells to seed. The medium used was Minimum Essential Medium (MEM) (+ 1 % p/s + 10 % FBS). From the fourth time, the medium was changed completely and reagents to enhance the cell growth were added. The medium composition was MEM (+ 1 % p/s + 10 % FBS), 1 % Non-Essential Amino Acids, 1 % GlutaMAX and 1 % Sodium Pyruvate. The medium was changed twice a week.

4.6.3 Quality Assessment Assays for MSCs

In order to validate the quality of the mesenchymal stromal stem cells extracted from the femoral bone, different assays were performed. The International Society for Cellular Therapy has described minimal criteria to define multipotent BMSCs in an attempt to foster a uniform characterization[129]. Therefore, the colony forming capability, the proliferation rate and the differentiation ability of the cells were analysed. Moreover, the expression of six cell-surface biomarkers were analysed using flow cytometry. Further sample analyses by mass spectrometry protein identification and genomic instability evaluation through Feulgen staining based image cytometry were also performed. An overview of the workflow is shown in **Figure 4.4**.

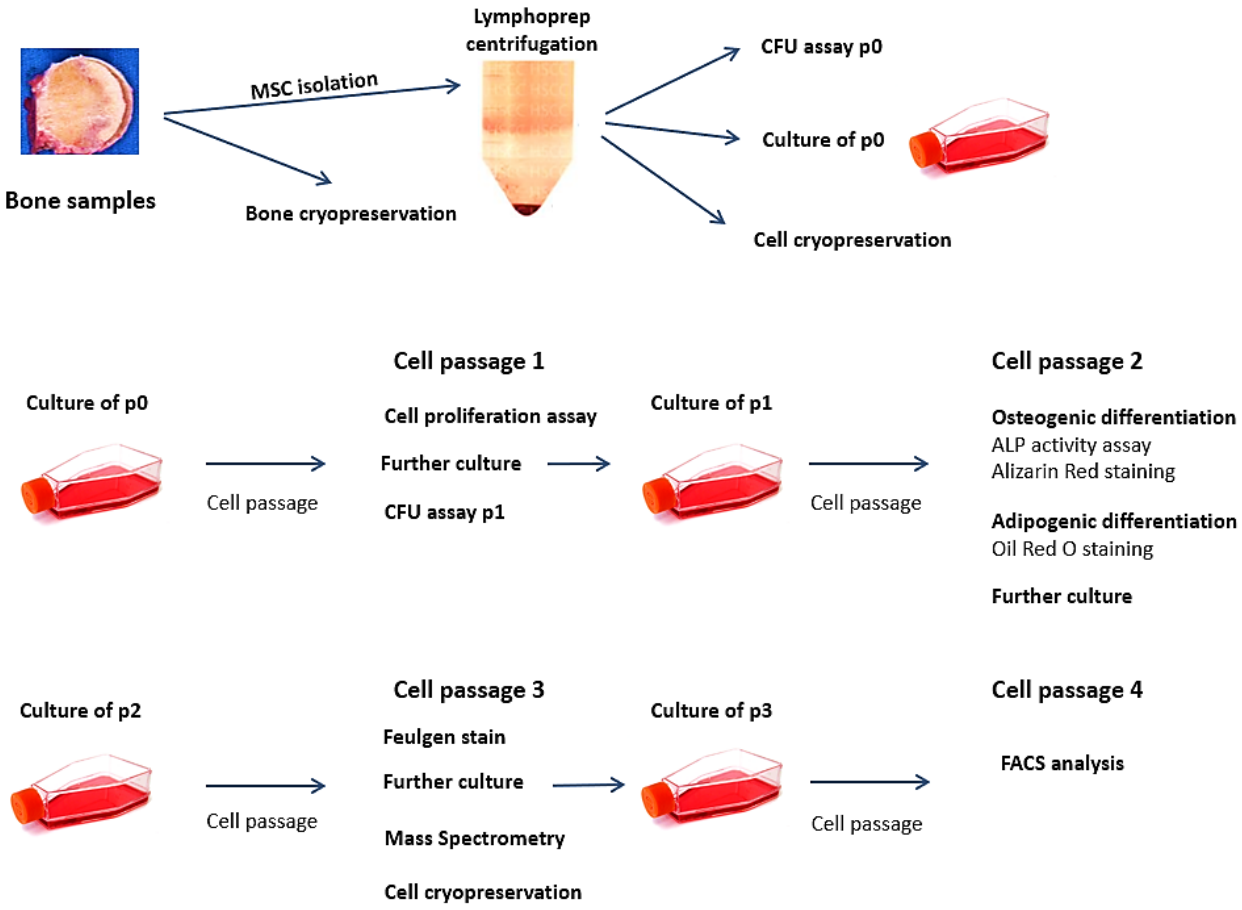

Figure 4.4 Schematic representation of the workflow for the cryopreservation study. Bone samples were used for the MSCs isolation, while a part was cryopreserved for further studies; the cells were divided in CFU assay, culture flasks and cryopreservation for further studies. Following the first passage of cells, the proliferation assay and a second CFU were performed, while a part of the cells was left in culture. At passage 2, the cells were divided for the osteogenic differentiation assays, adipogenic differentiation assay and further culture. Mass spectrometry sample preparation, Feulgen stain and cell cryopreservation were usually performed at passage 3, while the FACS analysis was accomplished at passage 4 of the cells.

4.6.3.1 Colony-Forming Unit Assay

The efficiency with which MSCs form colonies still remains an important assay for the quality of cell preparations, as one of their characteristic features is adherence to tissue culture plastic and generation of colonies when plated at low densities[126]. The assay was performed after 18 days of culture at passage 0 (1 x 10^6 cells) and again after 14 days at passage 1 (1,000 cells) in 3 Petri dishes (19.6 cm^2). The cells were stained with Crystal Violet dye diluted in DPBS (the solution was prepared with a ratio of 35 µl Crystal Violet and 700 µl DPBS per Petri dish) and incubated for 30 minutes with rotation at room temperature. Afterwards, the colorant was removed and several washes with DPBS were performed, to remove unspecific staining and the colonies were counted.

4.6.3.2 Proliferation Assay

The cell proliferation assay was performed to scrutinize the rate of cell proliferation. Proliferation is a fundamental property of stem cells, necessary for self-renewal and expansion and defining stem cell degree of stemness[201]. 180,000 cells were seeded into three 6-wells-plates at passage 1. This assay was performed in triplicate at day 1, 3, 6, 9, 12 and 15. The process involved the trypsinisation of the cells, which were then counted using Trypan blue and the Neubauer chamber. A mean of the results of the three wells was calculated and the results were plotted on a diagram to see the proliferation curve of the cells.

4.6.3.3 Osteogenic Differentiation

The cells were seeded at 20,000 cells/cm^2 in a 48- well plate for the evaluation of the osteogenic differentiation and in a 96-well plate for the quantification of Alkaline Phosphatase (ALP) activity. The induction was started at 90 - 100 % confluency. The cells in the wells designed for the osteogenic differentiation were fed with a medium composed of MEM medium (+ 1 % p/s + 10 % FBS), 5 mM β-glycerophosphate, 10 nM dexamethasone, 50 µg/ml vitamin C and 10 nM vitamin D_3, while the controls (undifferentiated MSCs) were fed with MEM medium (+ 1 % p/s + 10 % FBS). The medium was changed three times a week. The cells were cultured for 21 days before performing the evaluation of the osteogenic differentiation, accomplished with Alizarin Red S (AR-S), and the quantification of ALP activity.

4.6.3.3.1 Quantification of Alkaline Phosphatase Activity

Intracellular expression of ALP is very high in induced pluripotent and embryonic stem cells and therefore can be used as unique and unambiguous biomarker of stem cells[202,203].

The Alkaline Phosphatase activity assay can be done together with the Viability Assay, in order to normalize the ALP activity data to the number of viable cells. The CellTiter-Blue Cell Viability Assay uses the dye resazurin to measure the metabolic capacity of cells as an indicator of cell viability. Viable cells retain the ability to reduce resazurin (dark blue colour) to resorufin (pink colour), which is highly fluorescent ($579_{Ex}/584_{EM}$). Nonviable cells rapidly lose metabolic capacity, do not reduce the indicator dye and thus do not generate a fluorescent signal.

The master mix of the cell viability assay was prepared with a ratio of 20 µl CellTiter-Blue reagent to 100 µl of cell culture media per well. The cells were incubated in this new medium at 37°C for 1 hour. 60 µl of the cell viability assay media were then removed from each well and added to a dark 96-well plate and the fluorescent intensity was measured ($579_{EX}/583_{EM}$). Meanwhile, the cell viability assay was removed from the original plate and the wells were washed with TBS. The cells were fixed with 100 µl 3.7 % formaldehyde – 90 % ethanol for 30 seconds at room temperature and 100 µl of the

reaction substrate (1 mg/ml P-nitrophenylphosphate in $NaHCO_3$ 50 mM, at pH 9.6 with 1 mM $MgCl_2$) were added, followed by 20 minutes incubation at 37°C. Colour of the reaction buffer should change to yellow, due to ALP activity. After incubation, 50 µl of the stop reaction substrate (3N NaOH) were added. 100 µl of the solution were transferred to a new 96-well plate and the absorbance was measured at 405 nm.

4.6.3.3.2 Alizarin Red S Stain

Cells differentiated to osteoblasts can be stained for Alizarin Red S, which is a qualitative assessment of mineralized matrix formation[204–206]. The cells were washed with DPBS and fixed with 500 µl of 70 % ice-cold ethanol for 1 hour at -20°C. After fixation, the wells were washed with 500 µl Millipore and stained with 250 µl AR-S for 10 minutes at room temperature with rotation. The excess dye was washed with 500 µl water and DPBS with rotation for few minutes, in order to reduce non-specific Alizarin Red stain. To detach the staining from the cells, 125 µl of 10 % CPC were added to the wells and incubated at room temperature for 30 minutes with rotation. 20 µl of the solution were transferred in triplicates to a 96-well plate and mixed with 180 µl water. The intensity of the staining was measured at 570 nm.

4.6.3.4 Adipogenic Differentiation

The cells were seeded at 30,000 cells/cm^2 in a 24-well plate. The induction was started at 90 - 100 % confluency. The cells in the wells designed for the adipogenic differentiation were fed with a medium composed of DMEM medium (+ 1 % p/s + 10 % FBS), 5 % horse serum, 1 µM BRL, 3 µg/ml insulin, 100 nM dexamethasone and 225 µM IBMX, while the controls (undifferentiated MSCs) were fed with DMEM medium (+ 1 % p/s + 10 % FBS). The medium was changed three times a week. The cells were cultured for 14 days before performing the evaluation, which was accomplished with Oil Red O stain.

4.6.3.4.1 Oil Red O Stain

Cells differentiated to adipocytes can be stained for Oil Red O, which stains the accumulated cytoplasmic lipid droplets[207–209].

The dye solution was prepared as follows: 5 mg Oil Red O dye + 10 ml isopropanol + 6.7 ml H_2O. It was incubated at room temperature for 15 minutes and filtered through *whatman* paper before use. The cells were washed with DPBS and fixed with 1 ml of 4 % paraformaldehyde for 10 minutes at room temperature. Subsequently, the wells were washed twice with 1 ml of 3 % isopropanol and stained with 500 µl of Oil Red O for 1 hour at room temperature. The dye was then removed rinsing the cells with 1ml of water twice. In order to detach the staining, the wells were further washed with 1 ml of 3 % isopropanol and incubated with 120 µl of 100 % isopropanol for 10 minutes at room temperature.

100 µl of the solution were then transferred to a 96-well plate and the intensity of the staining was measured at 500 nm.

4.6.3.5 Flow Cytometry Analysis

Originally developed in the late 1960s, flow cytometry is a powerful tool for the analysis of multiple individual cell parameters from heterogeneous populations. Flow cytometry is used in several multicolour applications for biology or functional studies as well as a broad range of research applications, e.g. immuno-phenotyping, multi-parametric DNA analysis, proliferation, fluorescent protein and cell counting[210]. Thousands of cells per second pass one by one through one or more laser beams in a flow cytometer, which measures scattered lights (forward scatter (FSC) and side scatter (SSC)) at several angles and fluorescence signal emissions, which are detected with an optical collection system before amplification and electronic digitalization (**Figure 4.5**). Three parts constitute a flow cytometer: first, the fluidic system permits hydrodynamic focusing; second the excitation source and optical emission systems from the wavelength filters to the detectors constitute the optical part and finally the electronic system digitalizes the signal to be analysed with specific computer software.

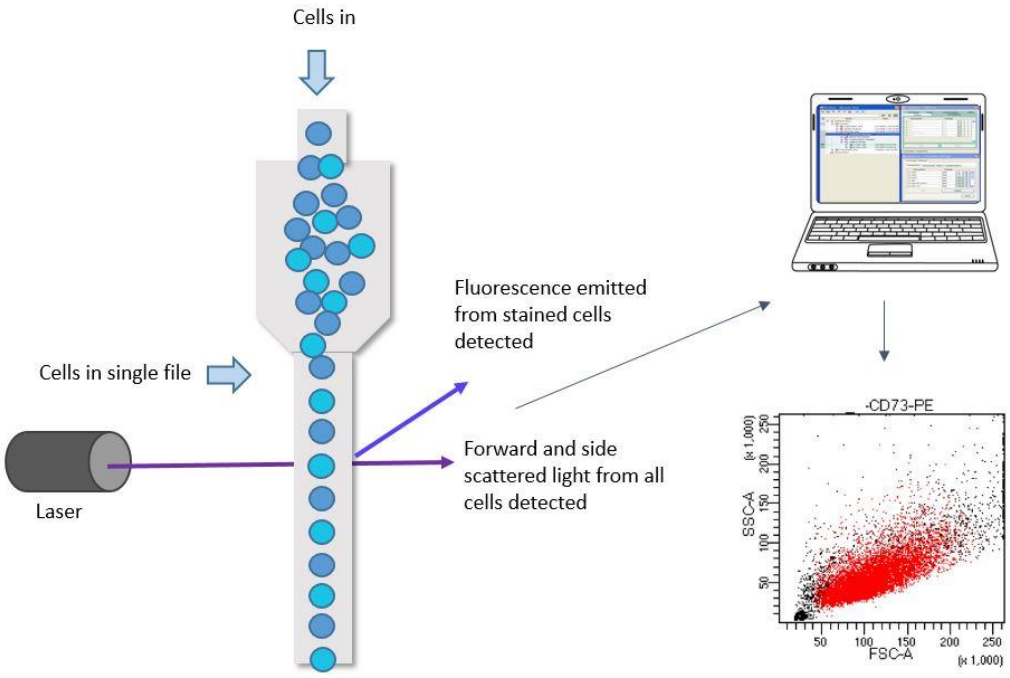

Figure 4.5 FACS mechanism and workflow. Sheath fluid focuses the cell suspension, causing cells to pass through a laser beam one cell at a time. Forward and side scattered light is detected, as well as fluorescence emitted from stained cells.

4.6.3.5.1 Fluorescence Labelling of the Samples

The cells were trypsinized, counted and resuspended in FACS buffer. The staining tubes were prepared with 10 µl of the specific fluorescent labelled antibody. A list of the antibodies and isotypes used is shown in **Table 4.5** and **4.6**. 100 µl of the cell solution, containing 100.000 cells, were added to the tubes, vortexed and incubated for 30 minutes in the dark at 4°C. 1 ml DPBS was added to wash unspecific antibody staining and the cell suspension was centrifuged at 1200 rpm for 10 minutes at 4°C. The liquid was discarded and the cell pellet was resuspended in 300 µl of the FACS buffer. Afterwards the tubes were vortexed, covered with aluminium and stored on ice before performing FACS.

4.6.3.5.2 FACS (Fluorescence-Activated Cell Sorting) Analysis

The analysis was performed right after the staining with the BD LSRII Bench Top Analyser (BD Bioscience, USA). FITC (Fluorescein isothiocyanate), PE (Phycoerythrin) and APC (Allophycocyanin) were used as fluorescence conjugates for the labelling of the antibodies. The three fluorochromes have the same Excitation length (488nm), however they have different Emission spectra (FITC at 519 nm, PE at 578 nm, APC at 660 nm). The 8-Peaks (Rainbow Particle) were used as internal control and contain a mixture of 3.0 - 3.4 µm Rainbow Particles that are dyed to eight different fluorescent intensities. Every Rainbow Particle contains a mixture of fluorophores that are excited at any wavelength from 365 - 650 nm. At least 10,000 cells were analysed per tube. The vitality of the cells was analysed using DAPI, with a final concentration of 0.1 mg/ml. 3 µl of the vitality dye were pipetted into the cell solution directly before the analysis. Exclusively samples with more than 70 % vitality were considered. The cytometric data were saved on a standard format (FCS) and analysed with the flow cytometry Software BD FACSDiva (BD Bioscience, USA).

Table 4.5 List of antibodies used for the flow cytometry analysis

Name	Conjugate	Clone	Host	Company
CD146	PE	Monoclonal	Mouse	Beckman Coulter
ALP	APC	Monoclonal	Mouse	R&D Systems
CD14	PE	Monoclonal	Mouse	BD Bioscience
CD34	PE	Monoclonal	Mouse	BD Bioscience
CD44	APC	Monoclonal	Mouse	BD Bioscience
CD73	PE	Monoclonal	Mouse	BD Bioscience

Table 4.6 List of isotype controls with the corresponding antibodies for the flow cytometry analysis

Isotype	Conjugate	Antibody	Company
IgG2a	PE	CD146	Beckman Coulter
IgG1	APC	ALP	R&D Systems
IgG2a, k	PE	CD14	BD Bioscience
IgG1, k	PE	CD34, CD73	BD Bioscience
IgG2b, k	APC	CD44	BD Bioscience

4.6.3.5.3 Representation and Analysis of the Flow Cytometry Data

The created dataset can be displayed in different formats. For the one-parameter representation, the intensity of the cell fluorescence is plotted on the X-axis and the number of positive cells is shown on the Y-axis. In the correlated two-parameter representation in the form of a "dot plot", in addition to the correlation of FSC with SSC, fluorescence of different wavelengths can also be correlated with one another or with scattered light properties. For the statistical evaluation, the number of defined events was determined as relative percentages. The percentage of marker-positive subpopulations was determined after exclusion of dead cells by so-called "gating" and in relation to the associated isotype control. The results were evaluated using the Flowing Software version 2.5.1.

4.6.4 Genomic Instability Assay

Genomic instability refers to a range of genetic alterations from point mutations to chromosome rearrangements and is usually associated with pathological disorders. From recent studies, it seems that genetic instability could also arise from prolonged cell passages, affecting cell growth and differentiation potential of the cells as well as reflecting increased for malignancy development [211–213].

4.6.4.1 Cytospins

Cytospins allow for uniform preparations of cells that are easily stained and evaluated[214,215]. The Cytospin centrifuge is designed to deposit cells evenly onto a glass slide and this is achieved by using a cytofunnel, which is attached to a glass slide and slide carrier. The entire apparatus is inserted into the cytocentrifuge and the cell suspension is added. The instrument produces a monolayer cell deposition in a defined area of the slide using centrifugal force. The cell suspension should be 10^5 cells/ml. For the analysis, cells were trypsinised and the suspension was centrifuged at 1880 rpm for 5 minutes at 19°C. The pellet was washed and resuspended in 2 ml DPBS. 100 µl of the suspension was loaded into each

cuvette and centrifuged at 500 rpm for 5 minutes. The cuvette and the paper were carefully detached and the cytospins were left to dry overnight before performing the Feulgen Stain.

4.6.4.2 Feulgen Stain

Air-dried cytospins were fixed in 4.5 % formaldehyde overnight. After washing under running water, acid hydrolysis in 5 M HCl for 1 h at room temperature was applied to split off selectively the DNA purine bases adenine and guanine resulting in the formation of none-purine acids with a free aldehyde group[216]. Cytospins were rinsed in Millipore water and treated in darkness with Schiff's reagent (stain: pararosaniline/fuchsin, Sigma-Aldrich, Germany) for 2 h at room temperature. During the reaction of the Schiff's reagent with free aldehyde groups, the hydrophobic, colourless fuchsin dye intercalates into the DNA. The subsequent charge transfer of the dye becomes visible as a red violet colour. After washing and incubation in fresh aqueous 0.005 M $Na_2S_2O_5$ solution 3 times for 10 min, Feulgen stained cytospins were rinsed under running water for 5 min, dehydrated in an ascending ethanol series (50 %, 70 %, 96 %, 100 %, xylene) and cover slipped using the mounting medium Entellan® (Merck KGaA, Germany). The intensity of Feulgen staining was measured using image cytometry.

4.6.4.3 Nuclear DNA Ploidy Measurements using Image Cytometry

Nuclear DNA ploidy determination was performed on Feulgen stained cytospins using image cytometry[217]. According to the absorption law of Lambert-Beer, the DNA content was calculated based on the measured optical density of the stained nuclear DNA. At least 500 non-mitotic nuclei per cytospin were selected interactively and the DNA content was determined semi-quantitatively using a computer-assisted imaging system equipped with a Nikon Eclipse Ci microscope and a 20x/0.40 plan objective (Nikon, Japan) in combination with ACAS software (Ahrens Cytometry Analysis System, Hamburg, Germany). Feulgen stained lymphocytes were applied as staining control with a given value 2c denoting the normal diploid DNA content. Relative DNA contents of all samples were calculated in relation to the corresponding lymphocyte reference and DNA profiles were classified according to Auer[218,219] (**Figure 4.6**). Histograms characterized by a single peak in or near the diploid region (1.5 – 2.5c) were classified as type I. Type II histograms represented a single peak in the tetraploid region (3.5 – 4.5c) or peaks in both, diploid and tetraploid, regions. Type III histograms exhibited highly proliferating cell populations near the diploid region and were characterized by DNA values ranging between the diploid and tetraploid region (2.5 – 3.5c). Thus, DNA histograms of type I, II and III represent diploid (euploid) cell populations. In contrast, type IV histograms showed enhanced and / or distinctly scattered DNA values exceeding the tetraploid region (> 4.5c). Hence, type IV histograms reflected aneuploid populations.

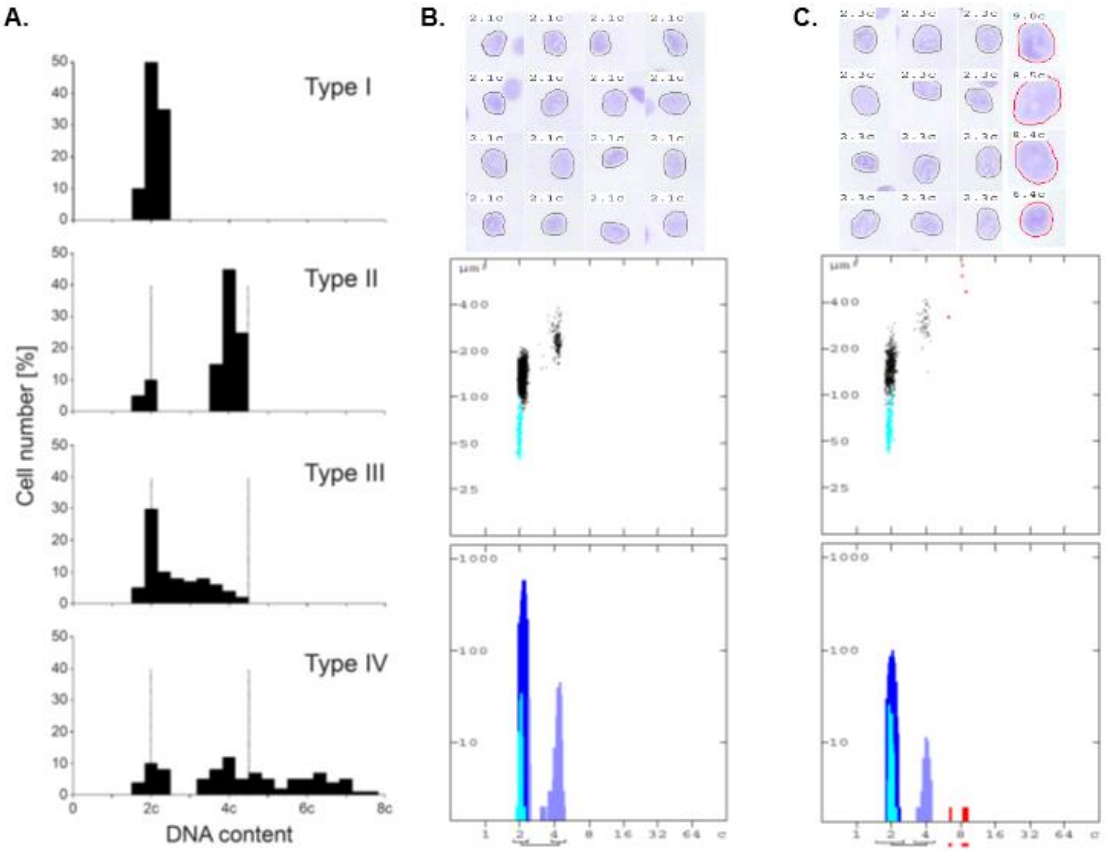

Figure 4.6 Genomic instability assessment according to Auer et al. **A)** DNA profiles of image cytometry are classified into type I – IV. Histograms of type I, II and III represent diploid (euploid) cell populations, whereas the type IV histogram reflects an aneuploid population with decreased genomic stability (Auer et al., 1980a; Auer et al., 1980b). Exemplary depictions of nuclear DNA ploidy measurements using image cytometry in combination with ACAS software on Feulgen stained cytospins of **B)** type II, diploid cells with slight proliferation, characterized by a peak in the diploid region (1.5 – 2.5c) and one in the tetraploid region (3.5 – 4.5c) and **C)** type IV aneuploid cells with enhanced DNA values exceeding the tetraploid region (> 4.5c). Selections of nuclei with the corresponding DNA content, dot plots of nucleus sizes, and histograms of frequency distribution of the DNA content are shown (from top to bottom). Reference nuclei of lymphocytes with diploid sets of chromosomes (2c) are imaged in cyan.

4.7 Statistical Analyses

4.7.1 Statistical Analysis for Serum Proteomics

Statistical analyses on the CRCS chip and ELISA data were performed using IBM SPSS Statistics v24 and GraphPad PRISM v5.04 software. For two-group comparison, the data were calculated using one-tailed Mann-Whitney U test. A p-value < 0.05 was considered as significant. Cut-offs for dichotomization into healthy control or pancreatic cancer patient were based on maximal sensitivity or specificity values using ROC curve calculation. The correlation between age and sex and the serum levels of the markers as well as correlations between the single markers was assessed using Spearman's correlation

coefficient. The AUC for the combination of all markers was calculated using logistic regression analysis.

4.7.2 Statistical Analysis for Cells based Methods

Statistical analysis of cryopreservation data was performed using IBM SPSS Statistics v24 and GraphPad PRISM v5.04 software. CFU assay, Proliferation assay, ALP activity assay and differentiation assays (Alizarin Red and Oil Red stain) values of triplicates per case were averaged. For two-group comparisons, measured values were calculated using the Wilcoxon test. A p value < 0.05 was considered as significant. The correlation among age, sex and the different quality parameters (colony forming, proliferation, osteogenic and adipogenic differentiation ability and FACS markers) was assessed using Spearman's correlation coefficient. The FACS results were evaluated using the Flowing Software version 2.5.1.

5 Results

5.1 Pre-analytical Stability of Biomarkers

The effects of storage time and temperature on serum protein levels have been evaluated for C3a desArg, CD26, M-CSF and S100A11 by applying a multiplex biochip and for M2PK and IL-18 using ELISA assays to a pool of serum samples collected from healthy donors (n=5). One aliquot was used for the fresh serum analysis and the remaining 16 serum aliquots were stored at room temperature, -80°C or below -130°C both, after gradient freezing (-80°C/<-130°C) and after shock freezing (<-130°C). Serum samples were analysed directly without storage (0h) and after 3 days, 2 weeks, 3 months, and 1 year.

5.1.1 Impact of storage time and storage temperature on markers composed on the Biochip

Median concentrations of all evaluated proteins including group comparisons statistics are summarized in **Table 5.1** and shown in **Figure 5.1**. Fresh serum measurements showed the following medians: $median_{C3adesArg}$ 1,253.0 ng/mL, $median_{CD26}$ 624.4 ng/mL, $median_{M\text{-}CSF}$ 4.13 pg/mL and $median_{S100A11}$ 1.53 ng/mL. Storage at 20°C resulted in significant serum level changes for all markers after 1 year of storage ($p < 0.05$, **Table 5.1**). The concentration of C3a desArg and S100A11 decreased by 88.5% ($median_{C3adesArg}$ 144.2 ng/mL, $p = 0.004$) and 75.3% ($median_{S100A11}$ 0.37 ng/mL, $p = 0.002$) after one year of storage, respectively. In contrast, the protein levels of M-CSF and CD26 increased by 2,178.5% ($median_{M\text{-}CSF}$ 90.17 pg/mL, $p = 0.002$) and 17.8% ($median_{CD26}$ 736.0 ng/mL, $p = 0.019$), respectively. Comparing storage temperatures of -80°C versus <-130°C, the levels of CD26 remained stable throughout time. Furthermore, no statistical difference was detected when comparing fresh serum samples with those stored at -80°C storage ($median_{CD26}$ 638.3 ng/mL) and below-130°C storage after gradient ($median_{CD26}$ 633.9 ng/mL) or shock freezing ($median_{CD26}$ 614.6 ng/mL, **Figure 5.1a**). Particularly noteworthy were the results for C3a desArg, S100A11 and M-CSF comparing fresh samples with those stored at -80°C at below -130°C after gradient and shock freezing. For C3a desArg, a considerably decrease of 53.2% could be detected after three storage days, which was continued for the complete period under observation for -80°C ($median_{C3adesArg}$ 499.4 ng/mL, $p = 0.004$), after gradient ($median_{C3adesArg}$ 443.3 ng/mL, $p = 0.004$) and shock freezing below -130°C ($median_{C3adesArg}$ 387.1 ng/mL, $p = 0.004$). Additionally, it has been shown that C3a desArg protein levels were different between storage at -80°C and below -130°C after one year ($median_{-80°C}$ 499.4 ng/mL, $median_{LN2}$ 387.1 ng/mL, $p < 0.001$; **suppl. Table 1, Figure 5.1c**). Similarly, S100A11 was significantly decreased for all evaluated timepoints across all storage conditions compared to fresh serum ($median_{80°C} = 1.31$ ng/mL, $p = 0.004$; $median_{-80°C/<-130°C} = 1.377$ ng/mL, $p = 0.027$; $median_{<-130°C} = 1.365$ ng/mL, $p = 0.037$, **Figure**

5.1d). Interestingly and, although presenting only minor absolute changes in comparing fresh and -80°C stored samples, M-CSF was significantly increased by 6.7% at -80°C (median$_{M\text{-}CSF}$ 4.417 pg/mL, p = 0.019). This protein did not reach significant changes at deeper temperatures (-80°C/<-130°C: median$_{M\text{-}CSF}$ 4.238 pg/mL; <-130°C: median$_{M\text{-}CSF}$ 4.239 pg/mL, **Figure 5.1b**) when compared to fresh serum.

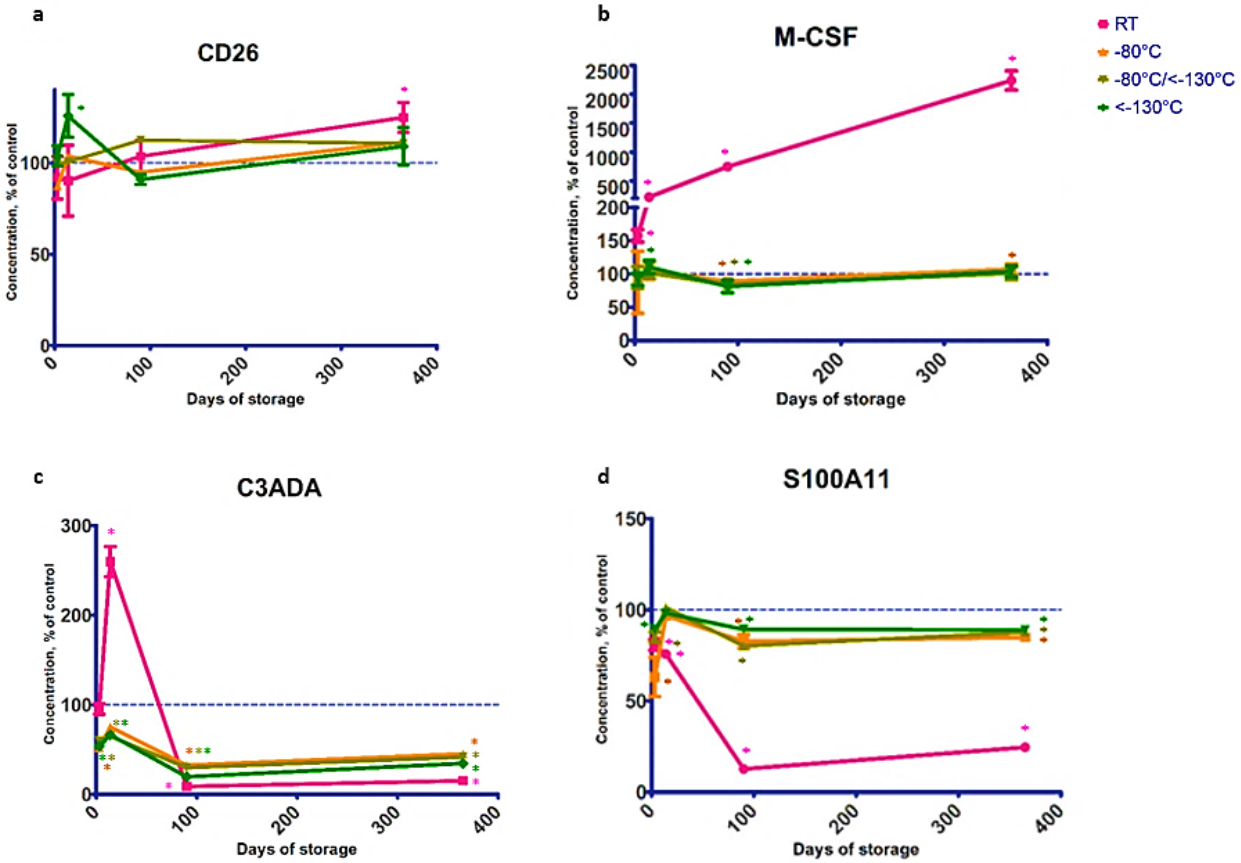

Figure 5.1 Kinetic diagrams of the four biomarkers subjected to different storage temperatures measured by biochip assay. Serum levels were measured at five different time-points (fresh, after 3 days, after 2 weeks, after 3 months, after 1 year) for each temperature condition (RT, -80°C, -80°C/<-130°C and <-130°C). The serum levels were calculated as percentage of the fresh serum concentration. The fresh serum levels are plotted as the reference (blue dotted line). * statistical significance ($p<0.05$) between fresh serum and respective sample aliquot at different storage temperatures and time points

5.1.2 Impact of storage time and storage temperature on markers assessed by ELISA

IL-18 showed a median concentration in fresh serum samples of 1.032 pg/mL. As IL-18 was not detectable any longer either after -80°C nor <-130°C storage, it was excluded from further analyses.

The distribution of measured M2PK serum levels including group comparison statistics is described in **Table 5.2** and shown in **Figure 5.2.** The median of M2PK in fresh serum was 7.378 ng/ml which decreased by 58.2% after one year of storage at room temperature ($median_{M2PK}$ 3.088 ng/mL, p = 0.004). Considering storage at -80°C and <-130°C, M2PK did show a significant increase of its concentration up to 225 % after one year of storage ($median_{-80°C}$ = 16.64 ng/mL, p = 0.002; $median_{-80°C/<-130°C}$ = 15.82 ng/mL, p = 0.009; $median_{<-130°C}$ = 14.15 ng/mL, p = 0.004). Additionally, the comparison between storage at -80°C and <-130°C showed a significant difference with lower concentration of M2PK found after storage at temperatures below -130° (p = 0.034; **suppl. Table 1**).

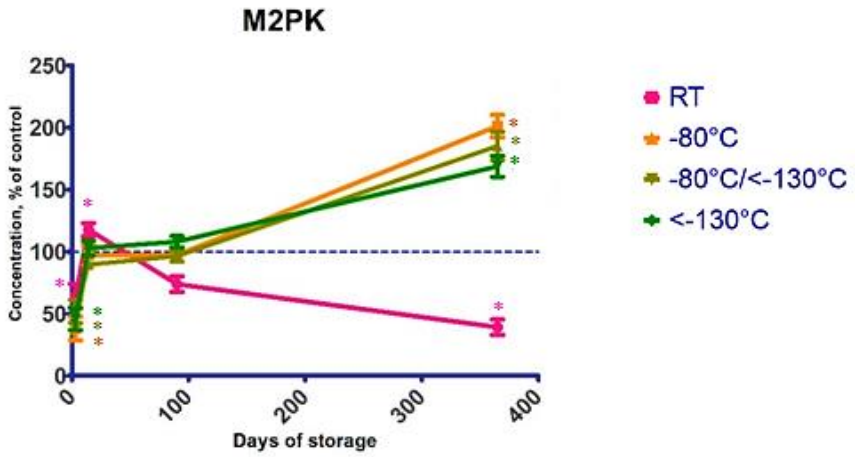

Figure 5.2 Kinetic diagram of the M2PK ELISA analysis subjected to different storage temperatures. Serum levels were measured at five different time-points (fresh, after 3 days, after 2 weeks, after 3 months, after 1 year) for each temperature condition (RT, -80°C, -80°C/<-130°C and <-130°C). The fresh serum levels are plotted as the reference (blue dotted line), * statistical significance ($p<0.05$) between fresh serum and respective sample aliquot at different storage temperatures and time points

Table 5.1 Median serum levels including interquartile ranges of the four biochip biomarkers at different storage times and temperatures. The p-values are calculated between each temperature/time point and the fresh serum measurement.

		CD26 (ng/ml)				M-CSF (pg/ml)			
Temp		**20°C**	**-80°C**	**-80°C/<-130°C**	**<-130°C**	**20°C**	**-80°C**	**-80°C/<-130°C**	**<-130°C**
fresh	**Median**	624.4				4.139			
	Interquartile Range	546.4 – 673.2				4.027 – 4.346			
3 days	**Median**	549.5	549.5	549.5	549.5	6.507	4.343	3.678	3.779
	Interquartile Range	474.2 – 580.2	474.2 – 580.2	474.2 – 580.2	474.2 – 580.2	6.275 – 6.809	4.247 – 4.884	3.460 – 4.544	3.691 – 3.953
	p-value	p = 0.130	p = 0.130	p = 0.130	p = 0.130	**p = 0.002**	p = 0.078	p = 0.078	p = 0.066
2 weeks	**Median**	502.8	502.8	502.8	502.8	8.940	4.216	4.188	4.575
	Interquartile Range	448.0 – 553.3	448.0 – 553.3	448.0 – 553.3	448.0 – 553.3	8.615 – 9.635	3.874 – 4.776	3.989 – 4.452	4.230 – 4.857
	p-value	p = 0.105	p = 0.105	p = 0.105	p = 0.105	**p = 0.002**	p = 0.375	p = 0.375	**p = 0.048**
3 months	**Median**	595.7	595.7	595.7	595.7	30.51	3.604	3.502	3.321
	Interquartile Range	542.5 – 625.5	542.5 – 625.5	542.5 – 625.5	542.5 – 625.5	29.83 – 32.35	3.424 – 3.855	3.202 – 3.578	3.029 – 3.718
	p-value	p = 0.492	p = 0.492	p = 0.492	p = 0.492	**p = 0.002**	**p = 0.009**	**p = 0.009**	**p = 0.006**
1 year	**Median**	736	736	736	736	90.17	4.417	4.238	4.239
	Interquartile Range	668.1 – 752.7	668.1 – 752.7	668.1 – 752.7	668.1 – 752.7	87.16 – 99.11	4.257 – 4.678	3.890 – 4.435	3.983 – 4.967
	p-value	**p = 0.019**	**p = 0.019**	**p = 0.019**	**p = 0.019**	**p = 0.002**	**p = 0.019**	p = 0.375	p = 0.322
		C3a desArg (ng/ml)				**S100A11 (ng/ml)**			
Temp		**20°C**	**-80°C**	**-80°C/<-130°C**	**<-130°C**	**20°C**	**-80°C**	**-80°C/<-130°C**	**<-130°C**
fresh	**Median**	1,253.0				1.532			
	Interquartile Range	1,010.0 – 1,276.0				1.455 – 1.688			
3 days	**Median**	1,115.0	549.2	619.1	591.8	1.259	1.233	1.317	1.381
	Interquartile Range	921.6 – 1,239.0	526.1 – 616.2	581.3 – 668.2	551.1- 619.9	1.115 – 1.343	1.108 – 1.323	1.207 – 1.411	1.321 – 1.460
	p-value	p = 0.130	**p = 0.006**	**p = 0.009**	**p = 0.006**	**p = 0.002**	**p = 0.008**	**p = 0.006**	**p = 0.037**
2 weeks	**Median**	2,715.0	801.4	707.9	714.5	1.162	1.497	1.541	1.514
	Interquartile Range	2,396.0 –3,277.0	735.7 – 902.1	697.1 – 732.0	686.7 – 767.9	1.100 – 1.243	1.453 – 1.560	1.494 – 1.653	1.452 – 1.578
	p-value	**p = 0.002**	p = 0.084	**p = 0.002**	**p = 0.009**	**p = 0.002**	p = 0.275	p = 0.275	p = 0.556
3 months	**Median**	99.2	354.7	304.6	210.6	0.182	1.296	1.225	1.388
	Interquartile Range	91.4 – 103.8	338.3 – 386.4	291.6 – 377.9	210.6 – 233.2	0.155 – 0.240	1.083 – 1.414	1.193 – 1.316	1.320 – 1.487
	p-value	**p = 0.002**	**p = 0.004**	**p = 0.004**	**p = 0.015**	**p = 0.002**	**p = 0.014**	**p = 0.014**	**p = 0.019**
1 year	**Median**	144.2	499.4	443.3	387.1	0.378	1.31	1.377	1.365
	Interquartile Range	140.6 – 191.2	477.9 – 518.8	435.3 – 483.3	362.1 – 397.8	0.351 – 0.416	1.234 – 1.408	1.305 – 1.452	1.275 – 1.493
	p-value	**p = 0.004**	**p = 0.004**	**p = 0.004**	**p = 0.004**	**p = 0.002**	**p = 0.004**	**p = 0.004**	**p = 0.037**

Table 5.2 Median serum levels including inter quartile ranges of M2PK ELISA analysis at different storage times and temperatures. The p-values are calculated between each temperature/time point and the fresh serum measurement.

		M2PK (ng/ml)			
Temp		**20°C**	**-80°C**	**-80°C/<-130°C**	**<-130°C**
Fresh	**Median**	**7.378**			
	Interquartile Range	**6.032 – 9.236**			
3 days	**Median**	5.274	2.324	3.596	2.648
	Interquartile Range	3.041 – 6.242	1.660 – 3.750	2.402 – 6.460	1.830 – 6.020
	p-value	**p = 0.027**	**p = 0.004**	**p = 0.002**	**p = 0.004**
2 weeks	**Median**	9.104	8.378	7.292	8.016
	Interquartile Range	8.619 – 10.200	5.556 – 9.166	6.572 – 7.596	7.295 – 9.468
	p-value	**p = 0.048**	p = 1.000	p = 0.492	p = 0.769
3 months	**Median**	5.154	7.638	7.434	8.452
	Interquartile Range	4.995 – 7.587	7.128 – 8.552	6.973 – 9.007	7.791 – 9.710
	p-value	p = 0.084	p = 0.922	p = 0.845	p = 0.375
1 year	**Median**	3.088	16.640	15.820	14.150
	Interquartile Range	1.962 – 4.550	14.150 – 18.480	13.470 – 16.800	11.540 – 14.910
	p-value	**p = 0.004**	**p = 0.002**	**p = 0.009**	**p = 0.004**

5.2 Pre-analytic Impact for Clinical Biomarker Research

A comprehensive evaluation of the impact of storage on four serum proteins (C3a desArg, CD26, M-CSF and S100A11) was performed on a sample cohort of 472 pancreatic cancer and 66 healthy control samples from seven clinical sites. The aim of the study was to verify if the storage could have an influence on the biomarkers' level and therefore on diagnostic accuracy. Two multiplex biochips were applied for this scope. Their performance for detecting pancreatic cancer was compared to markers used in clinical routine: Cyfra, CEA and CA19-9. Serum collection SOPs across clinical sides were comparable except for the fact that storage of samples was conducted either at <-130°C or -80°C. Additionally, an internal standard was applied in triplicates to each biochip run, to ensure - next to calibrations and quality controls - that the samples were not subjected to batch effects.

5.2.1 Evaluation of Single Markers and Combinations in the overall cohort

The distribution of the markers is described in **Table 5.3**. On individual analysis level, only Cyfra, CEA, and CA19-9 discriminated between pancreatic cancer cases and healthy controls ($p < 0.001$, $p = 0.001$, $p < 0.001$, respectively; **Figure 5.3**). Cyfra had a cut-off level of 1.95 ng/ml (79.8 % sensitivity and 81.1 % specificity), CEA of 2.05 ng/ml (66 % sensitivity and 66.7 % specificity), and CA19-9 of 32.25 U/ml (82.5 % sensitivity and 90.2 % specificity). The area under the curve (AUC) of the single markers are listed in **Table 5.5**. The two largest AUCs were observed for Cyfra and CA19-9 (0.858 and 0.879, respectively). It was then evaluated whether the combination of all markers indicates an advantage in terms of increasing the overall AUC compared to AUC of the single markers. This was assessed first in the overall sample cohort independent of the storage temperature. When considering the biochip markers, a statistical significance was found for the combination of CD26 and S100A11 with an AUC of 0.650 (sensitivity 95.1 % and specificity 31.2 %; $p = 0.002$). For the three routine diagnostic markers, the combination of Cyfra and CA19-9 resulted in a statistical significance with an AUC of 0.924 (sensitivity 90.2 % and specificity 86.3 %; p-value < 0.001). Lastly, when considering all the markers, a statistical significance was found when combining M-CSF, Cyfra and CA19-9 with an AUC of 0.941 (sensitivity 95 % and specificity 89.9 %; p-value < 0.001). The receiver operator characteristic (ROC) of the combined markers are shown in **Figure 5.5**.

5.2.2 Evaluation of storage temperature effects on marker's diagnostic performance

It was subsequently assessed whether the temperature of storage could influence the biomarker level concentration. When comparing pancreatic cancer samples stored <-130°C (n = 135) with pancreatic cancer samples stored at -80°C (n = 297), a statistically significant difference was found in the biomarkers' level for M-SCF and S100A11 ($p < 0.001$ and $p = 0.013$, respectively; **Suppl. Figure 9.1**).

Due to the low number of healthy controls stored at -80°C (n = 5), it was not possible to do the same comparison in the group of healthy control samples. When taking into consideration only the samples (diseased and healthy) stored <-130°C, serum levels of M-CSF and S100A11 showed statistical differences between pancreatic cancer patients and healthy controls ($p = 0.001$ and $p = 0.042$, respectively; **Figure 5.3**), while no statistical difference was detected for C3a desArg and CD26 ($p > 0.05$). M-CSF had a cut-off level of 5.56 pg/ml (66.7 % sensitivity and 67.6 % specificity) and S100A11 of 6.39 ng/ml (35.6 % sensitivity and 91.9 % specificity). The distribution of the serum levels is described in **Table 5.4**. Cyfra, CEA and CA19-9 also showed statistical differences between pancreatic cancer patients and healthy controls ($p < 0.001$, $p < 0.001$, $p < 0.001$, respectively; **Figure 5.4**): Cyfra had a cut-off level of 2.05 ng/ml (77.1 % sensitivity and 91.9 % specificity), CEA of 2.35 ng/ml (61.2 % sensitivity and 78.4 % specificity), and CA19-9 of 32.6 U/ml (85.3 % sensitivity and 94.4 % specificity). Furthermore, an increase in the AUCs was detected in all the markers (**Table 5.5**).

5.2.3 Evaluation of Marker Combinations in Samples stored <-130°C

Due to the differences of serum level concentrations dependent on the storage temperature as described above, the combination of markers was assessed also by considering only those samples stored <-130°C. This resulted for the biochip markers CD26 and S100A11 in an increased AUC of 0.724 (sensitivity 86.9 % and specificity 56 %; p-value < 0.001). For the three routine diagnostic markers, the combination of Cyfra and CA19-9 resulted in statistical significance with an increased AUC of 0.969 (sensitivity 97.2 % and specificity 88.3 %; p-value < 0.001). When considering all the markers, combining CD26, M-CSF, Cyfra and CA19-9 reached an unprecedented AUC of 0.987 (sensitivity 100 % and specificity 94.1 %; p-value < 0.001). The ROCs of the combined markers are shown in **Figure 5.5**.

5.2.4 Correlation among Biomarker Levels

In order to investigate whether the serum levels of the seven markers showed a correlation between each other, Spearman´s correlation coefficients were calculated. A relevant positive correlation was detected between M-CSF and S100A11 ($r_s = 0.412$). When analysing only the samples stored <-130°C, a relevant positive correlation was detected between C3a desArg and S100A11 ($r_s = 0.462$), between M-CSF and S100A11 ($r_s = 0.422$) and between Cyfra and CA19-9 ($r_s = 0.595$) (Spearman´s correlation coefficient > 0.40, $p < 0.01$) (**suppl. Table 9.8**).

5.2.5 Correlation between Biomarker Levels and Clinicopathological Features

Individuals in the control group were younger (59.7 years) than those afflicted with pancreatic cancer (65.8 years) ($p = 0.002$). Gender was distributed equally between the two groups ($p = 0.817$). In order

to explore whether the serum levels of the seven tested markers showed a correlation with age, gender, TNM status or grading, Spearman´s correlation coefficients were calculated. No relevant correlation was found. However, when considering only the samples stored <-130°C, a positive correlation was detected between serum levels of CA19-9 and grading (r_s = 0.542) and between serum levels of CA19-9 and M status (r_s = 0.533). No relevant correlation was found between marker serum levels and age or gender (Spearman's correlation coefficient > 0.40, $p < 0.01$) (**suppl. table 9.8**).

Table 5.3 Serum levels of seven serum biomarkers in pancreas cancer patients in comparison to healthy control patients.
‡ Comparison of serum levels between pancreas cancer patients and control patients $p < 0.05$.

		Storage at -80°C & <-130°C		**Storage at <-130°C**	
		Pancreas cancer patients (*n=431*)	**Healthy control patients (*n=42*)**	**Pancreas cancer patients (*n=135*)**	**Healthy control patients (*n=37*)**
C3a desArg ‡ (ng/mL)	Median	1,195.3	986.6	1,458.6	1,046.9
	inter-quartile range	738.25 – 2,140.85	654.4 – 1,569.9	727.4 – 2,613.8	686.8 – 1,573.05
	Range	123.4 – 16,319.2	396.2 – 5,871.2	123.4 – 16,195.80	396.2 – 5,871.2
	p-value	0.121		0.114	
CD26 ‡ (ng/mL)	Median	535.8	522.8	541.4	523
	inter-quartile range	389.1 – 805.2	415.6 – 588.9	410.7 – 890.5	412.75 – 593.05
	Range	122.4 – 2,295	215.4 – 1,123.2	122.4 – 2,132.4	215.4 – 887
	p-value	0.291		0.100	
M-CSF ‡ (pg/mL)	Median	4.97	4.43	6.13	3.981
	inter-quartile range	3.32 – 7.97	3.43 – 7.11	4.026 – 9.864	3.317 – 5.834
	Range	0.0 – 40.51	2.33 – 17.93	0.0 – 33.228	2.331 – 17.217
	p-value	0.517		**0.001**	
S100A11 ‡ (ng/mL)	Median	4.15	3.88	4.645	3.937
	inter-quartile range	2.98 – 6.09	2.93 – 4.81	2.988 – 7.786	2.989 – 4.84
	Range	0.11 – 26.54	2.04 – 7.68	0.536 – 22.201	2.098 – 7.686
	p-value	0.206		**0.042**	
Cyfra ‡ (ng/mL)	Median	2.8	1.2	2.95	1.2
	inter-quartile range	2.1 – 4.1	0.8 – 1.8	2.1 – 4.25	0.8 – 1.55
	Range	0.7 – 67.4	0.5 – 11.1	0.8 – 34.6	0.5 – 3.1
	p-value	**< 0.001**		**<0.001**	
CEA ‡ (ng/mL)	Median	3.0	1.75	2.85	1.7
	inter-quartile range	1.7 – 6.0	1.2 – 3.05	1.6 – 5.55	1.2 – 2.25
	Range	0.2 – 1,810	0.5 – 12.8	0.2 – 102.4	0.5 – 8.2
	p-value	**0.001**		**<0.001**	
CA19-9 ‡ (U/mL)	Median	186.0	12.6	187.5	12.35
	inter-quartile range	55.0 – 695.45	7.75 – 18.0	60.4 – 825.55	7.4 – 17.575
	Range	0.7 – 61,855	3.8 – 407.1	0.7 – 23,726.9	3.8 – 62.7
	p-value	**< 0.001**		**<0.001**	

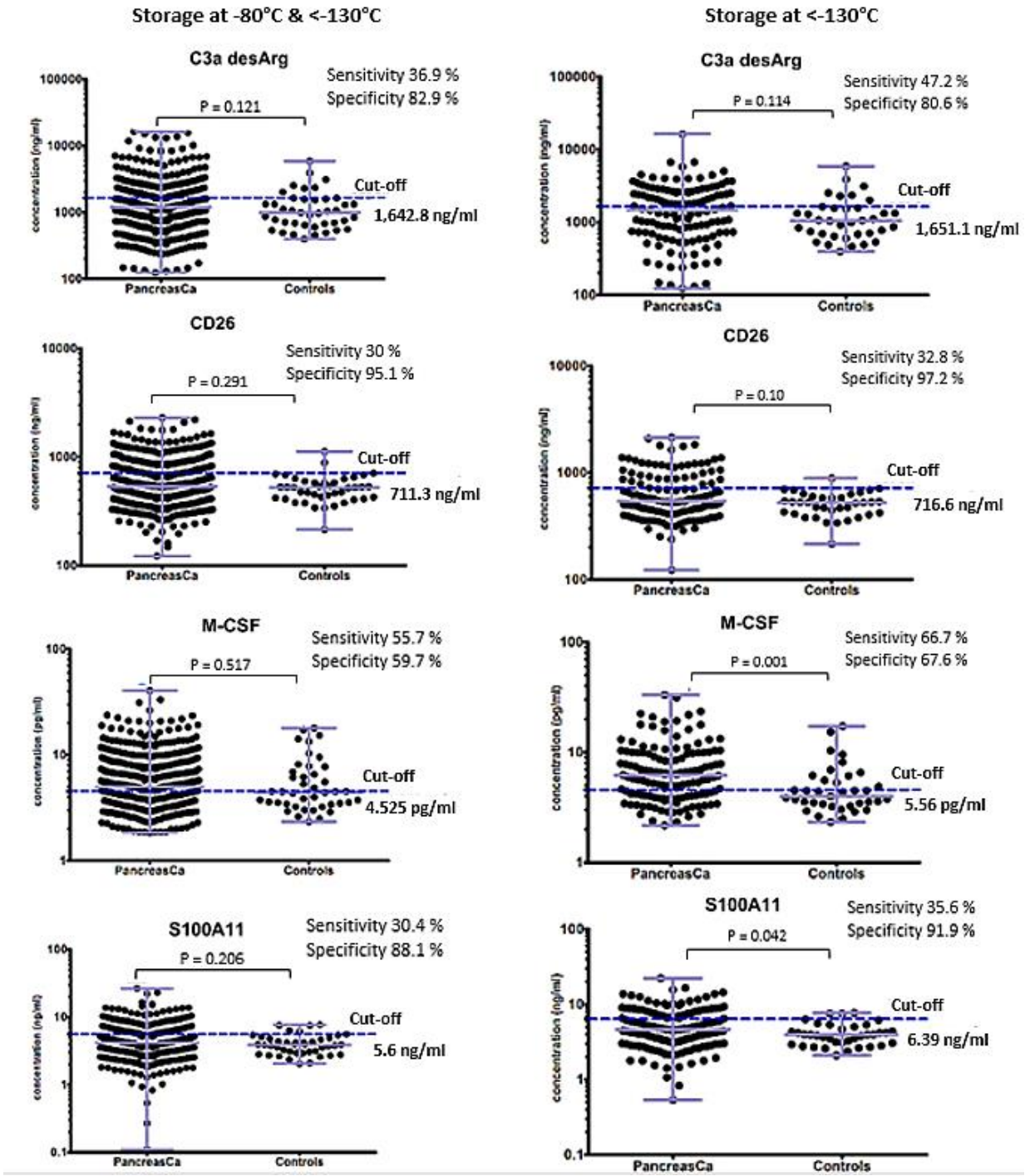

Figure 5.3 Scatter dot plots with the respective p-value, cut-off and sensitivity and specificity values of the single four biochip markers for pancreatic cancer and healthy control patients. The results of the scatter dot plots are represented in a logarithmic scale. M-CSF and S100A11 show statistically significant results when considering only the samples stored at <-130°C.

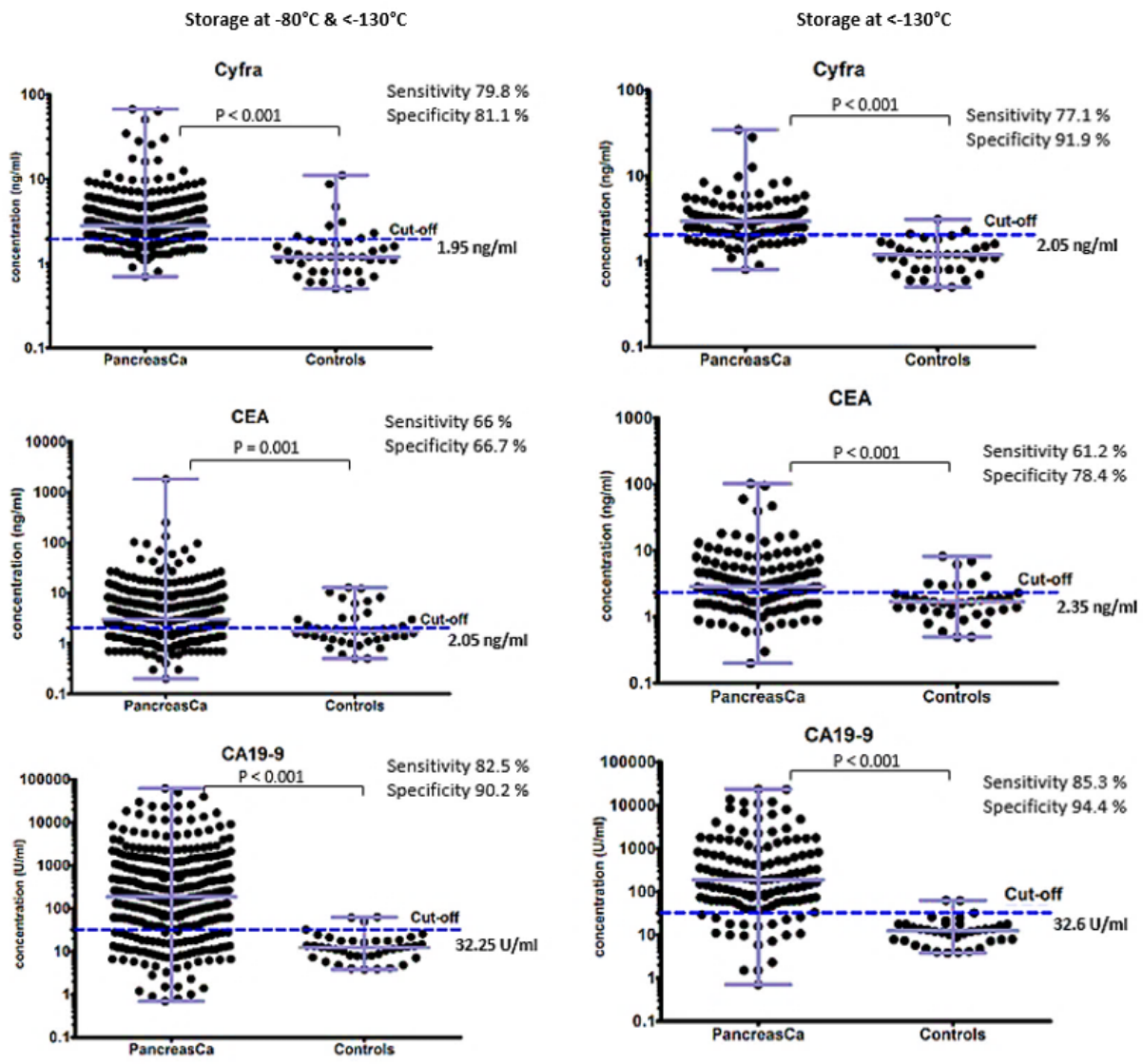

Figure 5.4 Scatter dot plots with the respective p-value, cut-off and sensitivity and specificity values of the single three routine diagnostic markers for pancreatic cancer and healthy control patients. The results of the scatter dot plots are represented in a logarithmic scale. All three markers show statistically significant results, both when considering all the samples and when considering only the samples stored at <-130°C.

Table 5.4 Area under the receiver operating characteristics curve (AUC) for all seven analysed biomarkers in single analysis.

	Storage at -80°C & <-130°C	**Storage at <-130°C**
Biomarker	AUC (95 % confidence interval)	AUC (95% confidence interval)
C3a desArg	0.573 (0.491 – 0.656)	0.587 (0.492 – 0.682)
CD26	0.550 (0.481 – 0.619)	0.590 (0.501 – 0.680)
M-CSF	0.530 (0.444 – 0.616)	**0.678** **(0.585 – 0.771)**
S100A11	0.559 (0.486 – 0.632)	**0.609** **(0.524 – 0.694)**
Cyfra	**0.858** **(0.782 – 0.935)**	**0.929** **(0.883 – 0.974)**
CEA	**0.661** **(0.576 – 0.746)**	**0.701** **(0.614 – 0.788)**
CA19-9	**0.879** **(0.839 – 0.919)**	**0.914** **(0.871 – 0.958)**

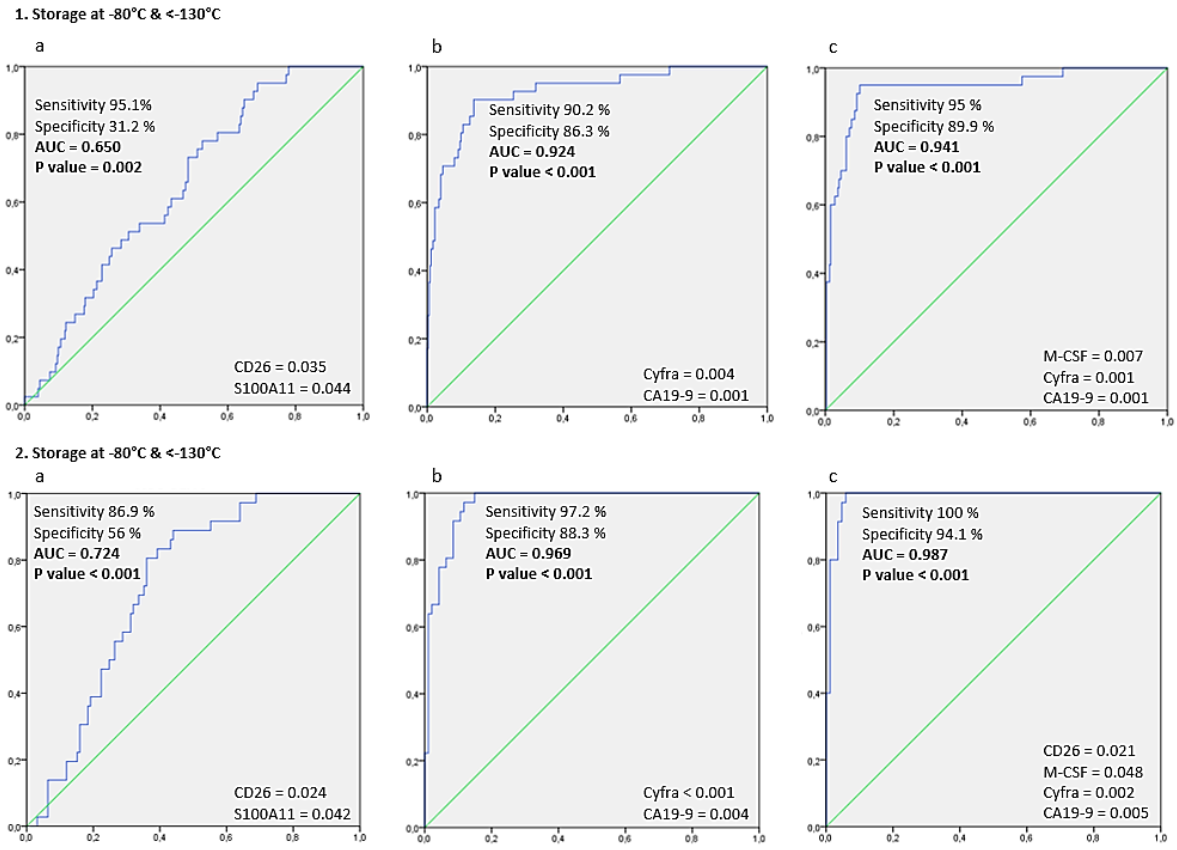

Figure 5.5 ROC (receiver operation characteristic) graphic representation (x = specificity; y = sensitivity) of **1a)** the four biochip markers (C3a desArg, CD26, M-SCF and S100A11), with a statistical significance for CD26 and S100A11 and an AUC (area under the curve) of 0.650 (95 % CI: 0.575 – 0.725); **1b)** the routine markers (Cyfra, CEA and CA19-9) with a statistical significance for Cyfra and CA19-9 and an AUC of 0.924 (95 % CI: 0.878 – 0.971); **1c)** all the seven markers with a statistical significance for M-CSF, Cyfra and CA19-9 and an AUC of 0.941 (95 % CI: 0.897 – 0.985). In the second part of the graph, ROC graphic representation (x = specificity; y = sensitivity) of **2a)** the four biochip markers (C3a desArg, CD26, M-SCF and S100A11), with a statistical significance for CD26 and S100A11 and an AUC of 0.724 (95 % CI: 0.644 – 0.803); **2b)** the routine markers (Cyfra, CEA and CA19-9) with a statistical significance for Cyfra and CA19-9 and an AUC of 0.969 (95 % CI: 0.943 – 0.994); **2c)** all the seven markers with a statistical significance for CD26, M-CSF, Cyfra and CA19-9 and an AUC of 0.987 (95 % CI: 0.971 – 1.000).

5.3 Mesenchymal Stromal Cells Quality Parameters Assessment

5.3.1 Cryopreservation

The effects of cryopreservation on MSCs quality parameters are reported by applying different assays (CFU assay, proliferation assay, osteogenic and adipogenic differentiation ability, and FACS markers expression) to 24 samples from eight patients. The MSCs directly isolated and cultivated (fresh condition) were used as a reference for the two cryopreserved conditions (cells extracted from the frozen bone or cells isolated and subsequently cryopreserved).

5.3.1.1 CFU & Proliferation Assays

The CFU assay was performed with cells at passage 1 and the colonies obtained were stained with crystal violet to visualise their total number. The number of the colonies varied between donors and between the three groups analysed, however there was no statistical difference in the amount of colonies among the three groups analysed ($p > 0.05$; **Figure 5.6**). The proliferation assay was also performed with cells at passage 1 to measure cell multiplication ability. The results indicated that cells isolated from the frozen bone and isolated and subsequently cryopreserved cells have a lower proliferation rate than "fresh" cells ($p < 0.05$; **Figure 5.7**). A statistical difference was detected between fresh cells and cells extracted from the frozen bone from day 6 onwards and between fresh and frozen cells from day 12 onwards.

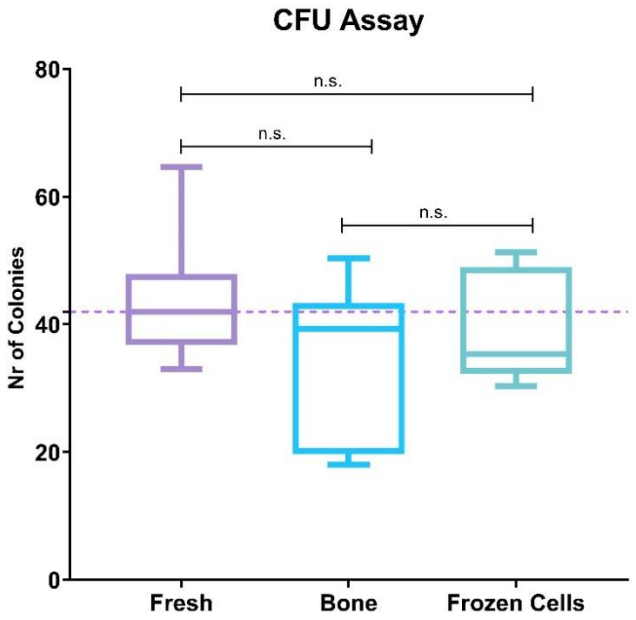

Figure 5.6 Results of colony forming unit assay among "fresh" cells (purple), cells isolated from the frozen bone (blue) and isolated and subsequently cryopreserved cells (green). No statistical difference was observed in the amount of colonies among the three groups analysed.

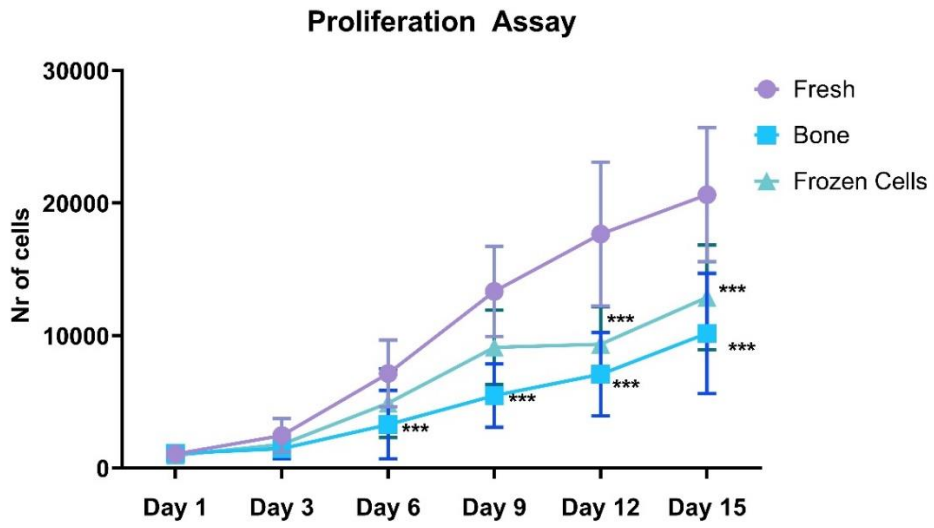

Figure 5.7 Results of the proliferation assay among "fresh" cells (purple), cells isolated from the frozen bone (blue) and isolated and subsequently cryopreserved cells (green). A statistical difference was detected between fresh cells and cells extracted from the frozen bone from day 6 onwards and between fresh and frozen cells from day 12 onwards.

5.3.1.2 In vitro Osteogenic Differentiation

5.3.1.2.1 Alizarin Red Stain

This osteogenic differentiation assay was performed with cells at passage 2. The osteogenic differentiation was assessed with Alizarin Red stain after 21 days of culture. The results showed that independent of the cryopreservation approach, cells were able to differentiate into osteoblasts. Interestingly, there was no statistical difference between "fresh" and cryopreserved cells in their potency to differentiate , even though cells isolated from the cryopreserved bone indicated a weaker staining compared to "fresh" cells (**Figure 5.8**). Instead, a statistical difference was observed between both cryopreserved conditions ($p = 0.0313$, **Figure 5.9A**).

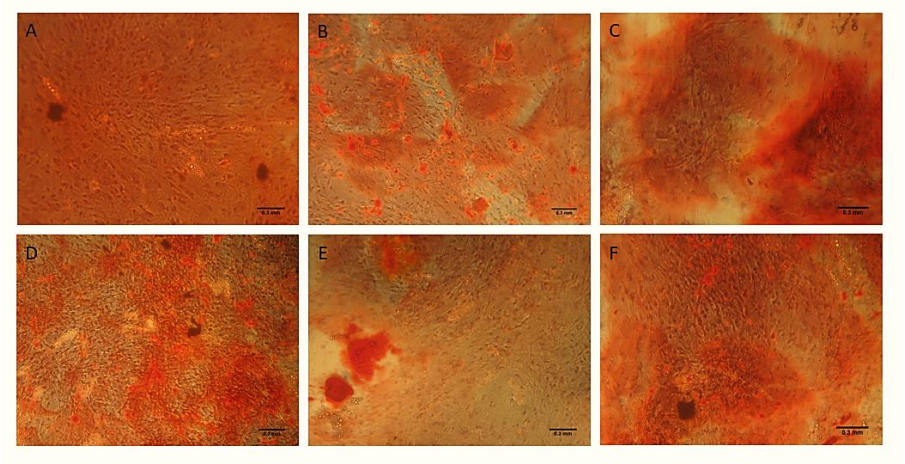

Figure 5.8 Two examples of Alizarin Red stain from two different patients after 21 days of culture in osteogenic medium, 10x magnification, size bar = 0.3 mm. The pictures indicate a heterogenic staining comparing "fresh" cells (A,D), cells isolated from the frozen bone (B,E) and isolated and subsequently cryopreserved cells (C,F).

5.3.1.2.2 Quantification of ALP Activity

This osteogenic differentiation assay was performed with cells at passage 2. The osteogenic differentiation was assessed by quantification of ALP activity after 21 days of culture, which uses P-nitrophenolphosphate as substrate to make P-nitrophenol. No statistical difference was detected between fresh and cryopreserved cells in their ability to produce ALP ($p > 0.05$; **Figure 5.9B**). However, also here cells isolated from cryopreserved bone tended to shower weaker staining results.

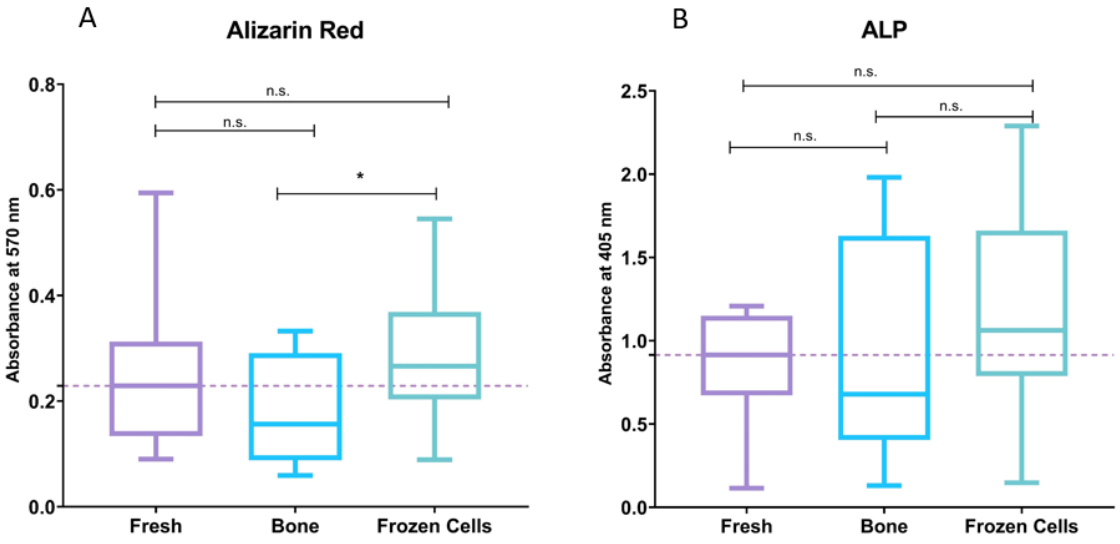

Figure 5.9 Osteogenic differentiation of MSCs among the three sampling conditions. Comparison of: **A)** Alizarin Red staining quantification among "fresh" cells (purple), cells isolated from the frozen bone (blue) and isolated and subsequently cryopreserved cells (green). No statistical difference was detected between "fresh" and cryopreserved cells in their potency to differentiate. A statistical difference was instead observed between cells isolated from the frozen bone and cryopreserved cells ($p = 0.0313$). **B)** Alkaline phosphatase (ALP) quantification among "fresh" cells (purple), cells isolated from the frozen bone (blue) and isolated and subsequently cryopreserved cells (green). No statistical difference was detected between "fresh" and cryopreserved cells.

5.3.1.3 In vitro Adipogenic Differentiation

This adipogenic differentiation assay was performed with cells at passage 2. The adipogenic differentiation was assessed with Oil Red O stain after 14 days of culture. The results showed that both cryopreserved groups were able to differentiate into adipocytes (**Figure 5.10**). Even though the quantification of the staining indicated a weaker staining in cells isolated from cryopreserved bone compared to the other two conditions, no statistical difference between "fresh" and cryopreserved cells in their potency to differentiate was detected ($p > 0.05$, **Figure 5.11**).

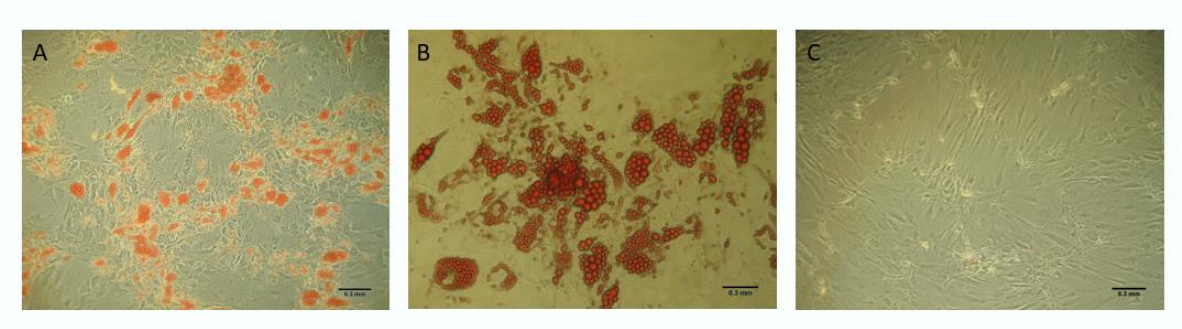

Figure 5.10 Adipogenic differentiation of MSCs. Example of **A)** Oil Red O stain after 14 days in adipogenic medium, 10x magnification, size bar = 0.3 mm; **B)** an enlargement of the lipid droplets, 20x magnification, size bar = 0.3 mm; **C)** control cells (undifferentiated MSCs), 10x magnification, size bar = 0.3 mm.

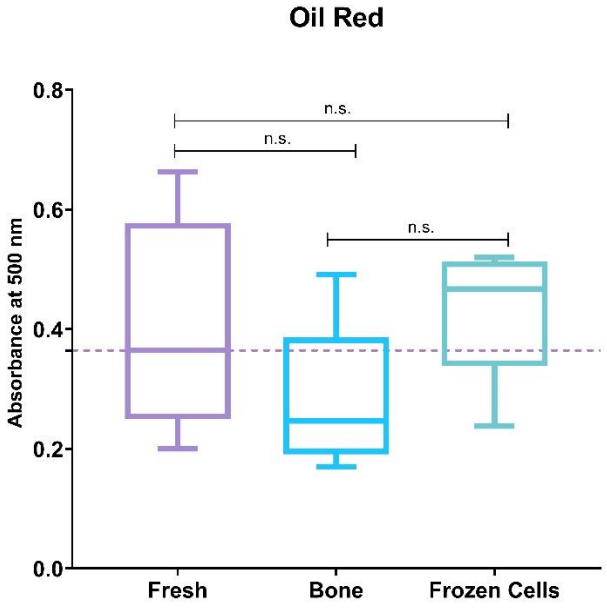

Figure 5.11 Comparison of Oil Red staining quantification among "fresh" cells (purple), cells isolated from the frozen bone (blue) and isolated and subsequently cryopreserved cells (green). No statistical difference was detected between "fresh" and cryopreserved cells in their potency to differentiate.

5.3.1.4 FACS Markers Expression

The FACS analysis was performed with the staining of six different markers: four positive (CD73, CD44, CD146 and ALP) and two negative markers (CD14 and CD34). Since the levels of the markers were evaluated with different runs over the course of several days, the 8-Peaks (Rainbow Particle) were used as internal control for the analysis. There was no statistical difference in the expression of the four markers in the three different sampling groups analysed ($p > 0.05$, **Figure 5.12**). According to the literature, the percentage of positive cells for CD73 and CD44 should be ≥ 95 %, while for CD34 and CD14 it should be ≤ 2 %. The majority of the measurements were in the correct range, however, there were some exceptions: For two patients the percentage of positive cells for CD73 were lower in cells extracted from frozen bone (94.6 % and 93.8 %) compared to fresh samples (99 % and 99.4 % respectively) and frozen cells (98.9 % and 98.5 %, respectively). Furthermore, for one patient the percentage of positive cells was lower than 95 % in both the fresh sample and the frozen cell sample (92.7 %). Also for CD44 there were some exception: For one patient the percentage of positive cells for this marker was lower in cells extracted from the frozen bone (94.6 %) than in fresh sample (99.8 %) and frozen cells (98.5 %). Furthermore, for one patient the percentage of positive cells was lower than 95 % in both the fresh sample (92.7 %) and the frozen cells (84.1 %). There were also some exceptions in the expression of CD34, where two frozen cell samples had higher percentage of positive cells for this marker (2.9 % and 8.1 %) than the fresh cells (0.5 % and 0.8 %, respectively) and cells from frozen bone (0 % and 0.1 %, respectively). For one patient both fresh and frozen cell samples had a higher level of this marker (72.4 % and 72.3 %, respectively), while for another patient only the fresh sample had higher values of this marker compared to the frozen cells ones (10.2 %). The number of cells positive for novel markers (CD146 and ALP) varied between donor samples (CD146 range: 0.1 % - 37 %; ALP range: 0.3 % - 13.7 %) and might therefore be associated with some of the biological functions of MSCs (**suppl. Figure 9.2**).

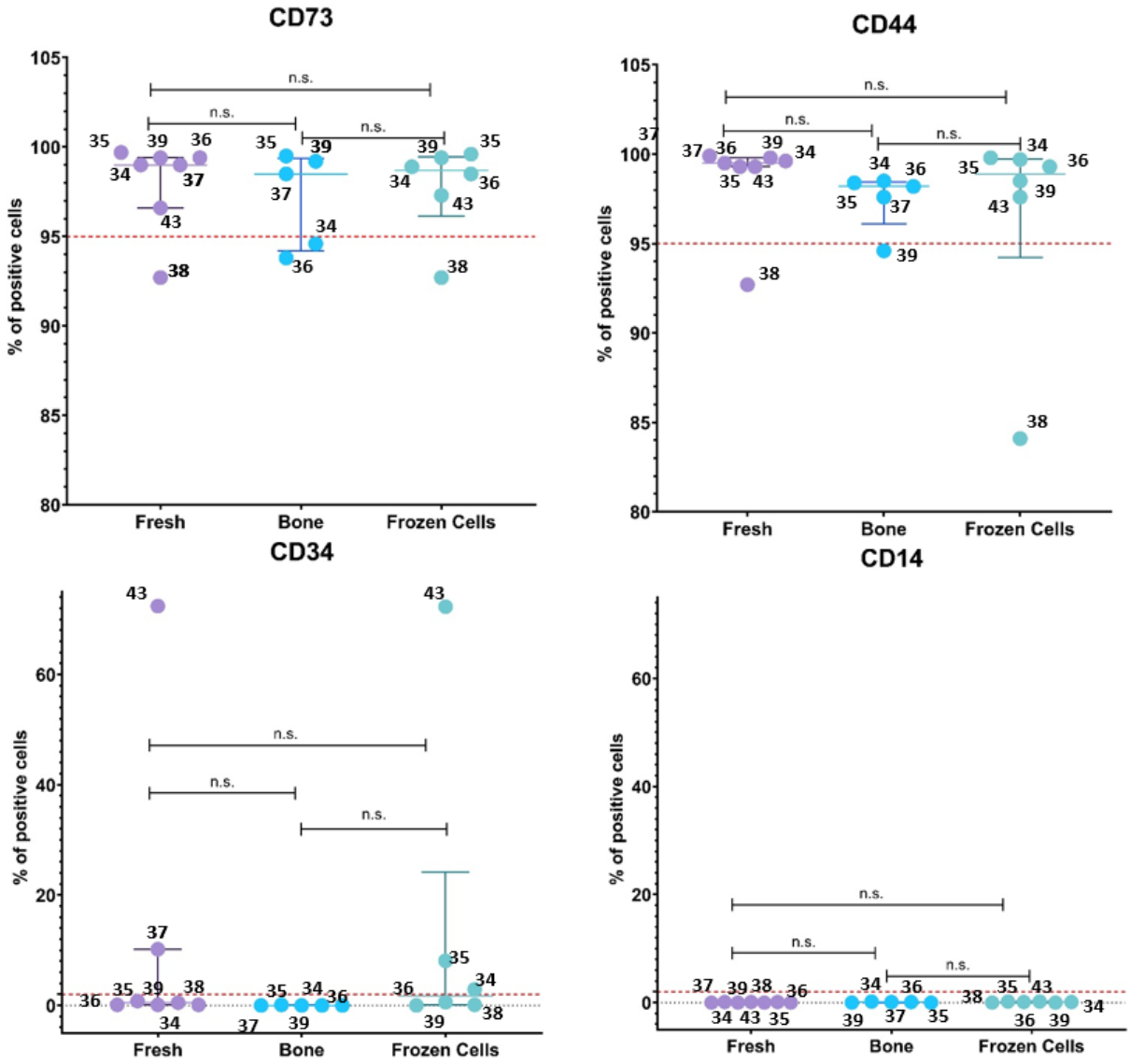

Figure 5.12 Box plot of the results of the FACS analysis among "fresh" cells (purple), cells isolated from the frozen bone (blue) and isolated and subsequently cryopreserved cells (green). The limit for positive cells were plotted and used as a reference for the analysis, therefore 95 % of positive cells for CD73 and CD44, and 2 % of positive cells for CD34 and CD14[129]. No statistical difference was detected among the three sampling groups for the four markers.

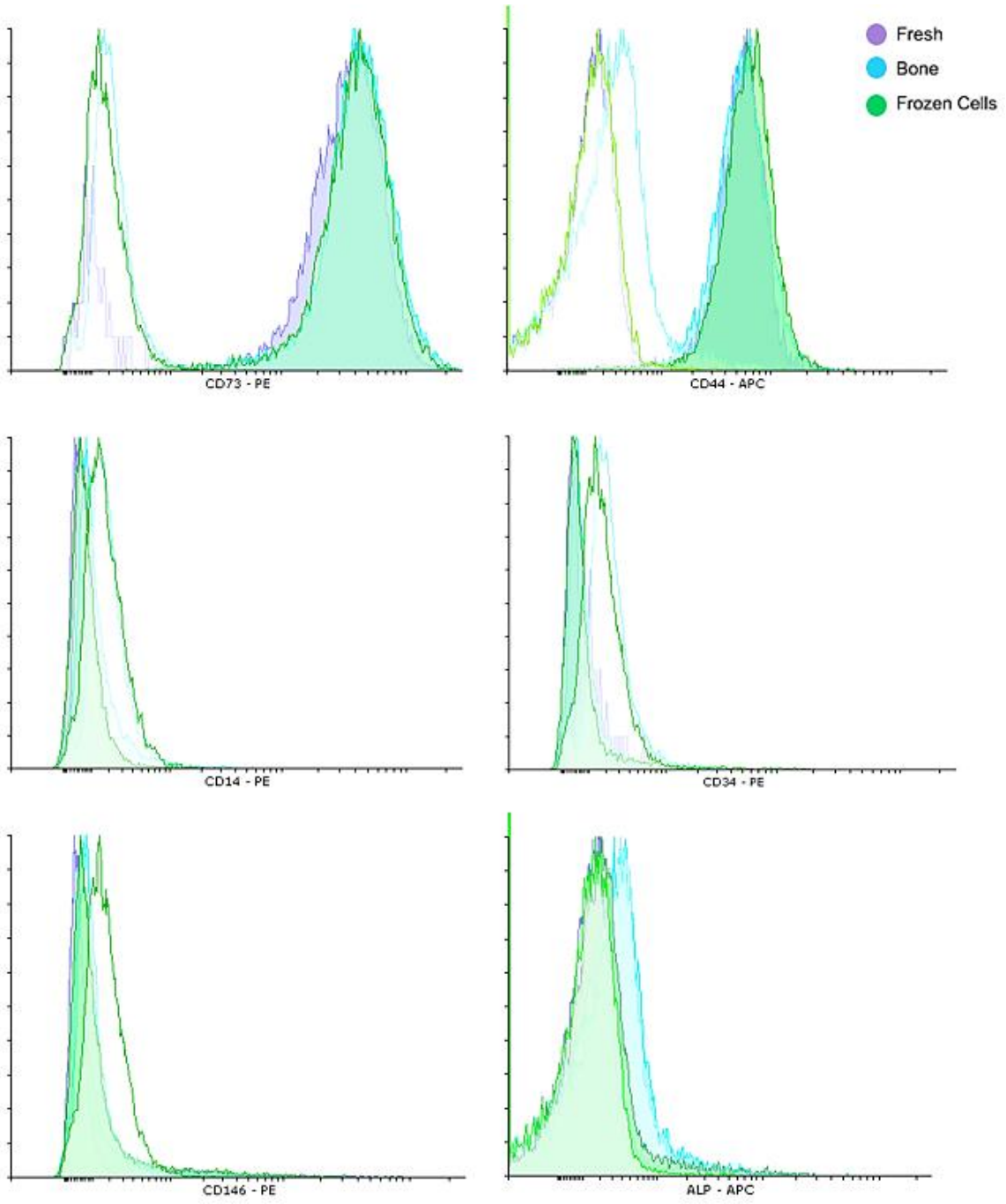

Figure 5.13 Exemplary FACS histograms from one patient for CD73, CD44, CD14, CD34, CD146 and ALP (full histograms). As a negative control, cells were incubated with the corresponding irrelevant mouse isotypes IgG1k, IgG2bk, IgG2ak, IgG2a, IgG1 (empty histograms). As expected, MSC were negative for reactivity to antigens CD14 and CD34, and positive for reactivity to antigens CD44 and CD73. Flow cytometry analysis demonstrated a heterogeneous MSC population for CD146 and ALP, with mainly low percentage of positive cells for these two markers (CD146 range: 0.1 % - 37 %; ALP range: 0.3 % - 13.7 %).

5.3.1.5 Correlation among Quality Parameters

In order to investigate whether the quality parameters of MSCs showed a correlation between each other, Spearman´s correlation coefficients were calculated. A negative correlation was detected between storage condition and proliferation (r_s = -0.649), storage condition and CD34 (r_s = -0.478),

time of culture and proliferation (r_s = -0.604), time of culture and CD73 (r_s = -0.624), time of culture and CD44 (r_s = -0.666), time of culture and CD34 (r_s = -0.565), proliferation and CD14 (r_s = -0.470) and ALP and CD73 (r_s = -0.583). This means that the fresh sample condition had the better proliferation rate and marker levels than the two frozen conditions, while the worst results were given by the frozen bone samples. The fresh samples were also the ones with the shorter time in culture, compared to the frozen cells and frozen bone conditions. Accordingly, a positive correlation was instead detected between storage condition and time of culture (r_s = 0.585), meaning that the time of culture increased with the type of storage condition; samples with longer time in culture were the cells extracted from frozen bone samples, while the fresh cells needed a shorter time in culture. A positive correlation was also detected between age and CD14 (r_s = 0.475), age and CD146 (r_s = 0.634), gender and CD73 (r_s = 0.499), CFU and proliferation (r_s = 0.566), CFU and alkaline phosphatase activity (r_s = 0.417), proliferation and adipogenic differentiation (r_s = 0.450), proliferation and CD34 (r_s = 0.525), alkaline phosphatase activity and osteogenic differentiation (r_s = 0.477), alkaline phosphatase activity and CD73 (r_s = 0.618), CD34 and CD44 (r_s = 0.452), CD146 and CD73 (r_s = 0.555), CD146 and CD44 (r_s = 0.505) and CD73 and CD44 (r_s = 0.518) (Spearman´s correlation coefficient > 0.40, $p < 0.05$) (**suppl. Table 9.9**).

5.3.1.6 Genomic Instability

The genomic stability of MSCs was also tested for all three sampling conditions. A cell suspension of 10^5 cells/ml was prepared from the cultured cells. The cells were fixed on cytospins and stained with Feulgen. Nuclear DNA ploidy determination was performed using image cytometry. No statistical difference was found among the three groups ($p > 0.05$). All samples analysed were diploid and the different sampling conditions applied did not seem to affect the genomic stability of the cell populations (**suppl. Figure 9.3**). In four patients (34, 38, 40 and 43), a low number of aneuploidy cells with a ploidy content above 5c was detected in all three conditions (fresh, frozen bone and frozen cells) and in only one patient (35) there was no aneuploidy present in cells of the three sampling conditions. In the remaining three patients (36, 37 and 39), the fresh samples did not present any aneuploid cells, while aneuploid cells with a ploidy content up to 9.5c appeared in low numbers in the two frozen conditions.

5.3.2 MSC Quality Score

Based on the results described above, a MSC Quality Score was created in order to classify collected samples for their overarching quality – and possibly clinical applicability - according to various quality parameters such as CFU, proliferation assay, differentiation ability and FACS marker expression. Here fore, each group of quality parameters was divided in quartiles and the samples were given a value from 1 to 4 according to which quartile the results belonged to. Therefore, the values above the 75th

quartile were given a score of 4, the values between the 75th quartile and the median had a score of 3, the values between the median and the 25th quartile had a score of 2, and the values lower than the 25th quartile had a score of 1. This was applied to the time of culture, CFU, proliferation, alkaline phosphatase activity as well as osteogenic and adipogenic differentiation. In contrast, values from 0 to 1 were assigned to results of the FACS markers: if the markers were in the range according to the literature (positive markers should be expressed by ≥ 95 % of the cell population and the negative markers should be expressed by ≤ 2 % of the cell populations) a score of 1 was given, otherwise a score of 0 was assigned. Only CD14, CD34, CD44 and CD73 were considered. According to this method, adding the results of the individual values would lead to the maximum score of 28, and the lowest score of 6. The mean of all the individual scores were plotted together and it was possible to detect a statistical difference among the three sampling groups according to the values of their final overall score (fresh vs bone, p = 0.0313; fresh vs frozen cells, p = 0.0313; bone vs frozen cells, p = 0.0313, **Figure 5.14**).

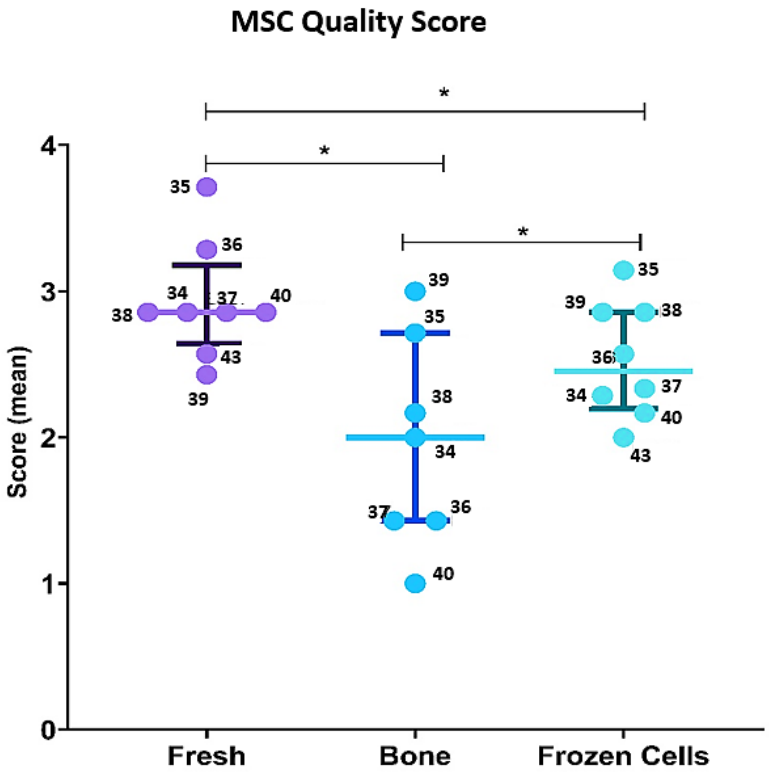

Figure 5.14 Box plot of the MSC Quality Score based on the evaluation of the MSCs quality parameters for "fresh" cells (purple), cells isolated from the frozen bone (blue) and isolated and subsequently cryopreserved cells (green). The mean of all the individual scores was plotted on the graph and it was possible to detect a statistical significant difference among the three sampling groups. The results showed an overall higher quality for the fresh cells compared to the two frozen conditions.

6 Discussion

This study focused on the importance of pre-analytical conditions for biomarker research studies and for the preservation of the quality parameters of cell samples. The main interest was to analyse different pre-analytical factors such as sampling, temperature and time of storage of serum samples and mesenchymal stromal (stem) cells (MSCs). Two important future clinical applications potentially biased by pre-analytics were chosen for this study. Due to the unique setting at the Campus Lübeck with its centralized hospital-integrated biobank infrastructure, it was possible to obtain, process and store serum samples and MSCs in a highly controlled fashion, and thus to analyse pre-analytical factors. These pre-analytical factors were chosen for their potential impact on the biomarker stability and quality of mesenchymal stem cell-based therapies. Pancreatic cancer was chosen as it remains one of the most lethal diseases with an increasing incidence in recent years[71]. Due to the inaccessible anatomical location of the pancreas as well as the lack of specific clinical markers[1], early detection of pancreatic cancer is still very difficult. MSCs were chosen as they have become quite popular in therapy applications over the years[72]. Their main application is in the repair of bone fracture, as musculoskeletal diseases remain among the most prevalent and challenging clinical problems, especially for the elderly population[73].

6.1 Pre-analytical Stability of Biomarkers

Many clinical samples are nowadays stored in freezers (-80°C) or <-130°C before they are used for e.g. supplementary molecular treatment decisions, blood transfusions, or biomarker studies. Although many potential sources like sample collection and processing[220,221] could impact the protein concentration critically, so far no study evaluated the influence of the initial freeze-cycle for sample biobanking. Especially the knowledge of the comparison between a direct determination in the clinical routine (“fresh samples”) and a determination of biobanked samples could lead to more successful biomarker studies. Against this background, the influence of storage temperature and time was examined on the serum protein concentration of six well known biomarkers (C3a desArg, CD26, M-CSF, S100A11, M2PK and IL-18) in comparison to freshly evaluated samples. In total, four different temperatures (20°C, -80°C, gradient freezing -80°C/<-130°C and snap freezing <-130°C) at five different time-points (fresh, three days, two weeks, three months and one year) were analysed using biochip and ELISA approaches. Ten replicates per aliquot of all temperature conditions were analysed in parallel in order to compare effects of storage temperatures on biomarker serum levels. Room temperature was chosen as a negative control for the analysis, while serum samples without any storage (fresh serum) were introduced as positive controls and as a new standard, as they fairly mimic the daily routine situation in clinical chemistry laboratories.

The results showed that the storage at room temperature affects the stability of all evaluable proteins overtime presenting either a significant concentration increase (CD26 and M-SCF, p = 0.019 and p = 0.002, respectively) or decrease (C3a desArg, S100A11 and M2PK, p = 0.004, p = 0.002 and p = 0.004, respectively). These changes could have been caused by a variety of different mechanisms, since degrading activities are not inert at room temperature and could therefore lead to increased protein proteolysis and other modifications[49]. Additionally, proteins can lose structural integrity and activity as a result of proteolysis and/or aggregation. No concentration changes – even for almost all results at room temperature – were observed for the glycoprotein CD26 which are generally considered as thermodynamically stable proteins[222].

By comparing fresh serum with storage at -80°C or <-130°C, we observed significant protein concentration changes for C3a desArg (p = 0.004 and p = 0.004, for -80°C and <-130°C, respectively), S100A11 (p = 0.004 and p = 0.037, for -80°C and <-130°C, respectively) and M2PK (p = 0.002 and p = 0.004, for -80°C and <-130°C, respectively). C3adesArg, also referred to as ASP (acylation-stimulating protein)[223], is a member of the complement system and represents the stable form of C3a anaphylatoxin in serum and plasma[224]. C3adesArg is an acute phase reactant, involved in a variety of biological functions[225,224,226]. S100A11 (also known as calgizzarin) is a member of the large calcium-binding S100 protein family that has been proposed to play specific biologic roles in the processes of endocytosis and exocytosis, enzyme activity regulation, cell growth, apoptosis and low-grade inflammation[227]. Most of S100 members exist in the form of antiparallelly packed homodimers (in some cases heterodimers), capable of functionally cross bridging two homologous or heterologous target proteins in a Ca2+-dependent (and, in some instances, Ca2+-independent) manner[228]. M2-pyruvate kinase is a glycolytic enzyme that catalyses the last step of glycolysis and mediates the transfer of phosphate from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP) to produce pyruvate and energy (ATP)[229]. While C3a desArg and S100A11 both resulted in lower protein levels after storage, M2PK showed an increase accumulation compared to fresh serum. C3a desArg was not associated with protein quality control and stability screening so far and there are not so many studies in the literature that analyse the effect of temperature on this protein. In just one study changes in the complement proteins levels after storage at -80°C were reported[230], with plasma samples yielding significantly higher concentrations with longer time in the freezer. The time the samples had been stored in the freezer before the measurement of biomarkers ranged from 6.6 to 10.6 years. In two other studies instead no significant effect of length of storage time was detected for complement proteins in plasma after 6 years at –80°C and after 10 days at 4°C, respectively[231,232]. In Bünger et al, there was no difference between -80°C and <-130°C storage, however the freeze-thaw cycles had an impact on the level of C3a desArg, with its level increased with repeated freeze-thaw cycles[233]. The same issue was observed for S100A11 and M2PK, where not so much literature was found for effects

of temperature. Another study reported no changes overtime in a calcium-binding protein, however a shorter time period was considered[234]. Hugo et al. stated that lymphocytes contain small amounts of the tumour M2PK isoenzyme and that they might release tumour M2PK in heparin-plasma and serum samples, but not in EDTA-plasma samples[235].

Unfortunately, in these studies the level of the proteins were not compared to fresh sample levels, but just with different time of storage and/or freeze-thaw cycles. For this reason, these different papers are not really comparable. One possible explanation for the behaviour of these proteins could be protein aggregation during storage. There are studies about this phenomenon, in which the conformational change in the protein structure causes an alteration in the assay detection[236,237].

M-CSF is a homodimeric glycoprotein, which could also explain its stability in storage overtime. M-CSF did not show any statistical change with the fresh serum levels after one year, when stored <-130°C. However, when stored at -80°C after one year its distribution showed a statistical change from the reference levels ($p = 0.019$). It can thus be supposed that this marker becomes unstable overtime when stored at -80°C, since as stated above, some degrading activities could still be active at this temperature and some analytes and proteins may be more affected than others[52,238,239]. It could be that some degrading activities changed the structure of this protein, causing also a change of the detection ability of the assay.

Interestingly, C3a desArg and M2PK also showed statistical changes between storage at -80°C and storage <-130°C after one year ($p < 0.001$ and $p = 0.034$, for C3a desArg and M2PK, respectively). This confirms the impact of the temperature of storage on different analytes and how their levels could change according to how they are stored. In the study of Bünger et al, even if no statistical difference between storage at -80°C and <-130°C was found, some patients would have not been detected as malignant for some markers if only storage at -80°C would have been considered. Furthermore, it has become clear that some degrading activities could still be active at this temperature and other studies already reported unstable results for different analytes stored in -80°C[50–52].

This study confirms the impact of pre-analytical conditions, such as temperature of storage, on biomarker stability. There are unfortunately only few reports on quantification of analyte degradation after long periods of storage and even when literature is available, results are not always consistent[240]. Furthermore, there is minimal literature focusing on measurement of fresh samples, since in most of the cases the samples are collected and stored before any analysis. However, the importance of a reference, like a “fresh” measurement, should not be underestimated. As shown by this study, without this measurement it would not have been possible to see the exact behaviour of these six markers overtime and in different storage temperatures. Thus, the fresh serum also would enable the identification of new quality markers, as it allows to verify which markers are more stable according to different temperatures of storage. To have this reference could be also of great importance for

multicentre studies, where usually the samples are stored for a certain number of years in different centres and biobanks; therefore allowing to detect other pre-analytical variables that could have influenced the analysis.

6.2 Pre-analytic Impact for Clinical Biomarker Research

The aim of this part of the study was to verify if the storage could have an influence on the biomarkers' level and therefore on diagnostic accuracy. A comprehensive evaluation of the impact of storage on four serum proteins (C3a desArg, CD26, M-CSF and S100A11) was performed on a sample cohort of 472 pancreatic cancer and 66 healthy control samples from seven clinical sites. Despite intense research efforts over the last decade, pancreatic ductal adenocarcinoma is still considered one of the most aggressive and lethal solid tumours. Notwithstanding its low incidence, it remains the fourth leading cause of cancer-related deaths in the modern world, mainly because of dismal diagnosis[241]. This is partly due to the fact that this type of cancer is at the beginning asymptomatic and there are no efficient screening assays as the technics that are currently used are either invasive or have low sensitivity[71]. The detection of C3a desArg, CD26, M-CSF and S100A11 in serum samples was achieved through the use of two multiplex biochips. Serum collection SOPs across clinical sides were comparable except for the fact that storage of samples was conducted either <-130°C or at -80°C. Additionally, an internal standard was applied in triplicates to each biochip run, to ensure - next to calibration and quality controls - that the samples were not subjected to batch effects.

Furthermore, confirmation was also given based on recent literature, since these markers have been already associated with pancreatic cancer in other studies. C3a desArg is a complement protein, has a molecular weight of 8.9kDa[242] and it has been reported to be identical to the acylation stimulating protein (ASP) and it is considered to be a metabolic bridge between events in the circulation and the adipocyte microenvironment[243]. It was already reported to be implicated in the early detection of colorectal adenomas and carcinomas[196], and it seems also to have a role in pancreatic cancer[244]. CD26 is a 110kDa type II transmembrane glycoprotein with dipeptidyl peptidase IV (DPPIV) activity in its extracellular domain and was shown to play a critical role as tumour suppressor or activator in different types of cancer[245–248]. M-CSF is a homodimeric glycoprotein of 85kDa[249] and is also involved in immunological and inflammatory reactions, bone metabolism and pregnancy[249,250]. Furthermore, it seems to play a functional role in tumour progression to metastasis[251]. S100A11 (also known as calgizzarin) is a member of the large calcium-binding S100 protein family that has been proposed to play specific biologic roles in the processes of endocytosis and exocytosis, enzyme activity regulation, cell growth, apoptosis and low-grade inflammation[252]. It seems to be influencing proliferation of the cancer cells[253].

Further markers analysed in the multicentre study for their role in the diagnosis of pancreatic cancer were Cyfra, CEA and CA19-9, since they are markers used in the clinical routine[254,255,121]. Cyfra 21-1 is a cytokeratin 19 fragment and is a member of the keratin family of intermediate filament proteins responsible for maintaining the structural integrity of epithelial cells[256]. Carcinoembryonic antigen (CEA), a glycoprotein with a molecular weight of 180 – 200kDa, was initially isolated from foetal colon and colon cancer tissue in 1965[257]. CEA is increased in various types of cancer, including colorectal cancer, breast cancer, lung cancer, and thyroid cancer[258]. Moreover, the serum level of CEA is increased in 30 % – 60 % of pancreatic cancer patients. Carbohydrate antigen 19-9 (CA19-9) is the sialylated Lewis blood group antigen originally defined by the monoclonal antibody 1116 NS 19-9[259,260]. Approximately three-quarters of all patients with pancreatic cancer have an elevated serum CA19-9 level at baseline. The results of this work showed that the temperature of storage had an impact on the serum marker concentrations. The importance of this variable is already discussed in the literature, as changes in serum and plasma biomarkers levels after different modalities of storage have been already documented[37,47,49,233]. Furthermore, in the study of Bünger et al, even if overall no statistical difference between storage at -80°C and <-130°C was found, on individual sample level some patients would have not been detected as malignant for some markers if only storage at -80°C would have been considered[261]. In this study, it was possible to observe that for M-CSF and S100A11 there was a statistical difference between pancreatic cancer samples stored at -80°C and <-130°C, suggesting that these two markers could be more sensible to the storage temperature ($p < 0.001$ and $p = 0.013$, respectively). When analysing all the samples, only Cyfra, CEA and CA19-9 resulted in statistically significance between pancreatic cancer patients and healthy controls ($p < 0.001$, $p = 0.001$ and $p < 0.001$, respectively). However, when analysing only the samples stored <-130°C, also M-CSF and S100A11 were statistically significant ($p = 0.001$ and $p = 0.042$, respectively), together with Cyfra, CEA and CA19-9 ($p < 0.001$, $p < 0.001$ and $p < 0.001$, respectively). Three different regression models were applied to improve the performance of the single markers: one model for the biochip markers, one for the routine markers, and one as combination of all markers. The best performance was obtained by the model composed of M-CSF, Cyfra and CA19-9, with 95 % sensitivity and 89.9 % specificity. However, when considering only the samples stored in nitrogen, the AUCs even improved and the best performance for pancreatic cancer was given by the combination of CD26, M-CSF, Cyfra and CA19-9 with an increased sensitivity of 100 % and specificity of 94.1 %. A combination of CA19-9 and M-CSF, together with CEA, was already reported in the literature for the diagnosis of pancreatic cancer with a sensitivity of XY and specificity of XY[262]. Moreover, Cyfra and CA19-9 have already been combined, together with other markers, for pancreatic cancer and cholangiocarcinoma in different studies, showing an improvement in their performance when compared to the single markers performance[263–265]. CA19-9 is the most widely utilized tumour marker for pancreatic cancer in the clinic, however it

faces also some controversies as predictive marker. CA19-9 is related to the Lewis blood group antigens and only patients belonging to the Le (α-β+) or Le (α + β-) blood groups will express the CA19-9 antigen[266]. Another challenge is that CA19-9 is also elevated in a variety of other conditions including malignancies such as cholangiocarcinoma, hepatocellular carcinoma, and colorectal adenocarcinoma as well as non-malignant processes such as pancreatitis, pseudocyst, choledocholithiasis, and cirrhosis. This could potentially affect its sensitivity and specificity for detecting pancreatic cancer in symptomatic patients[115]. Thus, the combination of CA19-9 with Cyfra, M-CSF and CD26 could increase the clinical performance of this marker and the detection of pancreatic cancer to a wider population, and not only to a group of patients. The combination of M-CSF and CA19-9 was already analysed with positive outcome by Groblewska et al[267]. The temperature of storage also had an impact on the correlations among markers. When analysing all the samples, a positive correlation was found only between M-CSF and S100A11. When analysing instead only the samples stored <-130°C, a relevant positive correlation was detected between C3a desArg and S100A11, between M-CSF and S100A11, between Cyfra and CA19-9, between serum levels of CA19-9 and grading, and between serum levels of CA19-9 and metastasis status. The correlation between M-CSF and S100A11 could be explained as these proteins are involved in inflammation responses and seem to be both involved in the activation and transport of macrophages during carcinogenesis[268]. Based on their function, macrophages are divided broadly into two categories: M1 (pro-inflammatory or 'classically activated' macrophages) and M2 (anti-inflammatory or 'alternatively activated' macrophages)[269]. In the presence of cancer cells macrophages differentiate into tumour associated macrophages (TAMs) which is a distinct subpopulation of M2 polarized macrophages. M2 macrophages will then enhance macrophage colony stimulating factors productions. In various forms of cancers, including pancreatic cancer, the presence of TAMs is associated with a worse prognosis[270]. In another study M-CSF was also reported to have increased levels in pancreatic cancer patients compared to healthy controls[271]. Regarding S100A11, the S100 gene is located on the chromosome region Iq21. Tumour genes in this region are frequently recombined, which may easily cause loss of S100 gene expression regulation. Other S100 protein family members like S100A10 seem involved in the activation or transport of M2 macrophages during cancer, therefore it could be hypothesised that S100A11 has the same functions, and therefore M-CSF and S100A11 are correlated, since as S100A11 increases, also macrophages M2 activation/production will increase and therefore more M-CSF protein will be produced[268]. Furthermore, it has been demonstrated that the expression of S100A11 is upregulated in the early stage of pancreatic cancer and decreases with the progression of pancreatic cancer[272]. The correlation between C3a desArg and S100A11, and between Cyfra and CA19-9 could be explained as for M-CSF and S100A11, since also these proteins are involved in inflammation responses. Therefore, in case of an inflammation state in the body, like cancer, these proteins will be increased or decreased according to their functions and

roles. It is known that S100 proteins are also involved in the immune response, working together with complement proteins, therefore it could be hypothesised that S100 proteins could also interact with complement protein C3a desArg during inflammation, in this case cancer[273]. Cyfra is a member of the keratin family responsible for maintaining the structural integrity of epithelial cells[256], while CA19-9 is a glycoprotein synthesized by pancreatic cancer cells as well as being produced by normal epithelial cells of the pancreas[274]. Therefore, it could be hypothesized that some changes during cancer can alter the keratin formation in cancer cells, which in turn could alter the epithelial cells and thus their production of CA19-9. CA19-9 and Cyfra were already combined for pancreatic and cholangiocarcinoma in different studies, and they always showed an improvement in their performances when combined. In the study of Nolen et al, a panel composed of Cyfra, CEA and CA19-9 provided significantly elevated sensitivity levels in pancreatic cancer patients, compared to sensitivity levels of CA19-9 alone[275]. In the study of Lumachi et al, the combination of CEA, CA19-9, Cyfra and MMP7 afforded a high sensitivity of 92 % and a high specificity of 95.8 % in detecting cholangiocarcinoma, showing the highest diagnostic accuracy, when compared to the single markers[264]. In the study of Chapman et al, a combination of Cyfra and CA19-9 resulted in sensitivity and specificity of 45 % and 96 %, while alone Cyfra had a sensitivity of 56 % and specificity of 88 % and CA19-9 had a sensitivity of 79 % and a specificity of 78 %[263].The correlation between CA19-9 and grading and M stadium could be explained, as according to the study of Hartwig et al, CA19-9 predicts resectability, stage of disease, as well as survival in patients with pancreatic adenocarcinoma. Highly elevated preoperative or increasing postoperative CA19-9 levels are associated with low resectability and poor survival rates, and demand the adjustment of surgical and perioperative therapy[105].

The results of this study prove that samples are subjected to the temperature of storage. Therefore, storing samples at a non-suitable temperature could impact their quality and fit-for-purpose for biomarker studies and, even more critical, diagnostic accuracy. In addition to the different storage temperatures (-80°C versus <-130°C), other factors could have influenced the analysis such as variations among the donors, the sampling, processing and transport of samples from one location to the other. However, due to the relatively high number of samples (-80°C = 297, <-130°C = 135), it seems unlikely that this statistical difference could only be casual. The results of this study are promising and show that the combination of the biochip markers with the routine markers could have a good clinical performance for the detection of pancreatic cancer. This could have positive implications for the diagnostic process to detect pancreatic cancer at early stage, since this of important clinical need since tests in current use are either invasive or have a too low sensitivity[71]. However, the impact of the temperature of storage should not be underestimated, as it could compromise multicentre studies.

In conclusion, careful planning should be applied for the collection and storage of biological samples, since pre-analytical variables could affect the results of experiments and therefore, compromise also diagnostic accuracy.

6.3 Mesenchymal Stromal Cells Quality Parameters Assessment

The third part of this study focused on mesenchymal (stromal) stem cells and their applicability for clinical studies. MSCs have gained a lot of popularity in therapy applications, as they possess a homing ability. This means that they can migrate into injured sites, and they possess the capacity to differentiate into local components of injured sites and the ability to secrete chemokines, cytokines, and growth factors that can "sense" the requirements of the environment[174]. These products can promote angiogenesis, regeneration, remodelling, immune cell activation or suppression, and cellular recruitment. At the same time the MSCs can also actively participate in bactericidal activity[174,175]. MSCs are great candidates e.g., for bone fracture healing. Musculoskeletal diseases remain among the most prevalent and challenging clinical problems, especially for the elderly population. Although simple fractures often heal effectively, the fracture healing process is impaired in 10 – 20 % of cases, causing non-union and severe disability[73]. Furthermore, some fractures, such as hip fractures, are threatening injuries with mortality rates of 15 – 25 %[181]. However, in the clinic it is not always possible to collect the bone material and isolate the cells on the same day. Thus, the aim of this study was to verify if different sampling methods could affect the quality of MSCs. Three different sampling methods were compared: a) direct isolation and cultivation of MSCs; b) storage of whole bone fragments <-130°C with subsequent isolation and cultivation of MSCs; and c) cultivation of MSCs after isolation and storage <-130°C. The bone and the cells were frozen with a standard freezing method, comprising a medium of 10 % DMSO and 90 % FBS and slow freezing method with *Mr Frosty* and storage <-130°C. The fresh collected cells were used as a reference for the analysis. The quality parameters of the three sampling conditions were analysed for 8 patients, on a total of 24 samples and included CFU, proliferation, differentiation ability (adipogenic and osteogenic differentiation) and FACS markers expression. From the results, there was no statistical difference among the three groups in their colony forming ability. There was however a lower amount of colonies in the cells extracted from the frozen bone, suggesting that the other two methods of sampling worked better and that the sampling method could have an impact on the colony ability of MSCs. Expanding this study for samples and patients would be needed to confirm this tendency and possibly reach statistical significance. The proliferation assay had instead a negative correlation with the storage condition, the cells being highly affected in their ability to proliferate. Therefore, a statistical difference could be detected between the fresh cells and the two other sampling methods. Also in this case, the cells extracted from the frozen bone had the least proliferation compared to the other two methods, as it could be seen already after six days

in culture, while the frozen cells started to show a statistical difference in their proliferation after nine days of culture. CFU and proliferation assay had a positive correlation among each other, meaning that the more highly proliferating cells also produced more colonies. A positive correlation was also found for storage condition and time of culture, confirming that the storage condition had an impact on the proliferation of the cells: cells extracted from frozen bone had the worst proliferating rate and needed longest time in culture, while the "fresh" condition was the fastest with the best proliferation rate. There was no statistical difference between fresh and frozen bone and between fresh and frozen cells in the osteogenic differentiation ability of MSCs. However, also in this case the cells extracted from frozen bone had the lower Alizarin Red staining intensity and therefore, were probably less differentiated than the cells of the other two groups. Osteogenic differentiation was highly heterogeneous among the patients even in the same sampling group, however the bone samples had in most of the patients analysed a weaker staining compared to the other two groups. The frozen cells showed a good differentiation potential, suggesting again that this sampling method could be better suited as replacement for the fresh condition. However, as mentioned already, it has been widely accepted that primary MSCs cultures are a heterogeneous population of cells with varying capacities of self-renewal and differentiation[122]. Therefore, also in the fresh conditions cells from some patients could not differentiate so successfully into osteoblasts. Alkaline phosphatase activity was less affected by storage, as it was possible to find highly metabolic cells in the three groups. However, the bone samples had again the lowest alkaline phosphatase values, with only two patients presenting highly metabolic cells, while the frozen cells had the highest one. This could have been a false positive, therefore more patients would be needed to analyse the effects of storage on alkaline phosphatase activity. Also CFU had a positive correlation with alkaline phosphatase activity, therefore it could be hypothesised that highly metabolic cells produced more colonies than non-metabolic cells. Alkaline phosphatase activity correlated positively also with osteogenic differentiation. Alkaline phosphatase is known to be essential for the process of mineralization, in which minerals such as calcium and phosphorus are deposited in developing bones and teeth[276]. Alkaline phosphatase activity is also used, together with Alizarin Red staining, as a quality parameter to ascertain if the osteogenic differentiation worked. Adipogenic differentiation was instead observed in all the three groups, with all the samples in the three groups being able to successfully differentiate into adipocytes. No statistical difference was found among the three groups, however also in this case the staining had lower intensity in the bone samples, signifying that less lipid droplets were produced in this group of cells. Since the cells from the frozen bone were proliferating slower, it could be hypothesised that they will also need more time to differentiate and produce lipid droplets and for this reason the staining intensity in this sampling group might have been lower. This was confirmed by the positive correlation between adipogenic differentiation and proliferation. Therefore, less differentiated cells should also have a

slower proliferation. This could create some controversies since proliferation and differentiation belong to two different parts of the cell cycle. However, the study of Brown et al. has made it clear that differentiation and cell proliferation are regulated simultaneously but independently, that cells often start differentiating long before they stop dividing, and that the launching of differentiation is not restricted to any particular segment of the cell cycle[277]. This combination of attributes allows expansion of cell numbers and acquisition of differentiated function to occur in parallel, generating abundant effector cells[277]. The sampling method did not seem to affect the FACS markers expression either, no statistical difference being found. Some outliers were instead detected, meaning that for some patients some of the markers were not in the right ranges according to the minimum criteria proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy[129]. There was however no correlation indicating that these outliers could be caused directly by the storage condition used. This phenomenon could have been caused instead by a later or earlier passage, or by some environmental factors, like medication of the patients. While we aimed to perform the FACS analysis always at the same cell passage, this was not always possible in particular for the frozen conditions since the cells were growing slower and therefore it was necessary to further passage them to get to the required number of cells for performing the FACS analysis. However, we could confirm that storage condition and time of culture had a negative association with CD34 marker expression, and CD73 and CD44 had a negative correlation with the time of culture. As mentioned above, many studies hypothesised that MSCs lose marker expression after extensive culture, and this is also the case for the marker considered to have a negative expression[278]. The lack of CD34 seems to be an effect of extensive culture, since CD34 is thought to be present in the early passages[279]. In this study, for one patient both frozen and fresh conditions had higher CD34 expression (more than 70 % of the cells expressed this marker). CD34 expression is almost ubiquitously related to hematopoietic cells, and it is common to consider CD34-positive cells in nonhematopoietic samples as a sign of hematopoietic contamination. However, there has been evidence that CD34 is expressed also by MSCs as by other non-hematopoietic cell types, and that the CD34 positive cells indicate a distinct subset of cells with enhanced progenitor activity[278]. Thus, this result could have been caused by either an earlier passage of these cells or it could mean that the cells from this patient had a higher amount of cells with progenitor activity. CD73 and CD44 also had a negative correlation with the time of culture, meaning that their expression decreases after extensive culture. It is known that MSCs are passage dependent and have a finite replicative capacity in culture[280]. From early passage numbers, MSCs begin to demonstrate senescence-associated changes such as reduced proliferative capacity, changes in morphology, shortening of telomeres, and an accumulation of the cyclin-dependent kinase inhibitor p16INK4a/CDKN2A. Within approximately 5 – 12 passages, MSCs demonstrate decreased clonogenicity and differentiation potential[281]. Thus, it could be possible that also the marker expression

will be affected by the time of culture. Furthermore, CD34 is also subjected to the storage condition, therefore cells extracted from the frozen bone had the lowest expression of this marker, while the fresh cells had the highest percentage, even if still in the right range. Since it seems that this marker is fundamental in the early passages for MSCs properties like colony forming and differentiation ability, its lack in the frozen bone, or lower percentage, could explain why this type of cells presented the lowest number of colonies compared to the other two conditions and the worst differentiation[282]. However, this assumption should be further confirmed by analysing the FACS markers at different passages of MSCs, to verify if differences in their expression could be detected. Proliferation also correlated positively with CD34 expression; therefore, it could be explained that proliferating cells should express more CD34 in the early passages. The proliferation assay seemed instead to have a negative correlation with CD14: the cells proliferated faster the less CD14 they expressed. There has been evidence that CD14 positive cells have shown features like slow proliferation, difficulty of passaging in the absence of growth factor support and differentiating in late phase[283]. Therefore, this could explain why MSCs expressing more of this marker are slower in proliferation also in this study. However, it is not clear how CD14 could affect the proliferation of the cells when it is more expressed. It could be connected to a CD14 immunosuppressing role, working together with Toll-like receptors (TLRs). CD14 acts as a co-receptor, exhibiting many characteristics of a pattern-recognition receptor, which is expressed mainly by macrophages, neutrophils and dendritic cells. Therefore, a lack of CD14 in MSCs may lead to 'partial activation' mode of the TLR4 signalling pathway, thereby inducing some specific gene expression[284]. CD73 correlated with gender, with women expressing higher levels of this marker. Nevertheless, this result should be interpreted carefully since there were more women in this study compared to men (5 females and 3 males). Therefore, this could have affected the results and more samples would be needed to validate this finding. Alkaline phosphatase activity correlated positively with CD73 expression. Ectoenzymes ecto-5'-nucleotidase (CD73) commonly serves to convert AMP to adenosine, and alkaline phosphatases are tissue-nonspecific enzymes, expressed on intestinal, placental, and germ cells, which also contribute to the metabolism of AMP to adenosine[285]. Therefore, this could explain the positive connection between CD73 and the levels of alkaline phosphatase in MSCs. Regarding CD146 and ALP, these are novel markers and in particular CD146 is considered by some authors as a new possible marker to distinguish mesenchymal stromal cells from fibroblasts[286,287]. The challenge with MSCs is in fact the difficulty to discriminate them from fibroblasts, since they have the same spindle shape, the same colony ability and express the same markers. In this study, CD146 and ALP were not so homogeneously expressed by the cells, and in most of the cases a really low number of cells were positive for these markers. It is important to keep in mind that MSCs are really heterogeneous and can variate also according to the tissue of origin. Thus, it could be that mesenchymal stromal cells extracted from the bone do not express these two markers, or express

them in lower quantity, while they are more expressed in bone marrow cells. Nowadays, many studies are focusing in finding specific markers for MSCs, also according to their tissue of origin. In the study of Sacchetti et al, CD146 was found to be a key marker for bone marrow MSCs[288]. However, in other studies it became clear that CD146 expression depended by the environment, even among bone marrow MSCs. Hence, this marker is heterogenous in the MSC population and highly dependent on tissue of origin and molecular environment[289,290]. In another study, it was verified that within the heterogeneous MSC population, cells have different degrees of maturity and therefore they differed in their expression of CD146. CD146 negative cells seemed to form more bone and less marrow and were thus more mature, whereas CD146 positive cells retained more plasticity, in addition to bone marrow formation, and they had a trans-endothelial migration capacity[291]. ALP is instead responsible for the regulation of ATP homeostasis in MSCs and subsequently lead to the fate switch in MSC differentiation and senescence[276]. A deficiency of this marker may therefore result in prototypical premature bone ageing characterized by bone mass loss and parallel marrow fat gain coupled with elevated p16INK4A (p16) and p53 expression[292]. Bone aging is partially orchestrated by ALP, which regulates the differentiation and senescence of MSCs. Mechanistically, ALP deficiency in MSCs results in enhanced ATP release and reduced ATP hydrolysis, which is, in turn, internalized by MSCs and consequently contributes to the cell fate change by regulating the AMPKα pathway[292]. Therefore, the explanation why these samples expressed low levels of this marker, could be that they are samples from older patients and that some of them have bone pathologies like osteoporosis. This could affect the number of cells expressing this marker. CD14 and CD146 were also positively correlated with age: the older the patient, the more of these markers were expressed by the cells. CD146 is a cell adhesion molecule (CAM) that is primarily expressed at the intercellular junction of endothelial cells[293] and is also involved in various physiological processes, including cell–cell and cell–matrix interactions, cell migration, and signalling, as well as morphogenesis during development[294]. Some of these properties could be more enhanced in older patients, explaining why this marker is more expressed. Regarding CD14 instead, older patients might express this marker more frequently, since CD14 seems to affect the proliferation and differentiation of the cells as mentioned above. Thus, it could be hypothesized that older patients will have less proliferating and differentiating cells. However, there was no correlation in this study between age and time of culture or differentiation ability; in some cases cells from older patients could differentiate even better than those from younger ones; thus, age seems not substantially affect the quality of the cells. This correlation could be more related to the clinical and medical conditions of the patients, for example which kind of medications they are taking, which in turn could enhance the expression of CD14. However, it should be noted that when mentioning that this marker is more expressed in the cells of older patients, it is still less than 2 %, since all the samples were negative for this marker and were always in the correct range according to the MSCs minimum

criteria. Regarding the correlations among the surface markers, a positive correlation was detected between CD34 and CD44, between CD146 and CD73, between CD146 and CD44, and between CD73 and CD44, while a negative correlation was found between ALP and CD73. In the study of Neu et al, an association between CD34 and CD44 was already reported, as CD34 positive cells were also expressing CD44. It is widely accepted that populations of CD34 positive cells are heterogeneous with regard to pluripotency and state of differentiation. The fact that CD44 has already been found on immature cells such as CD34 positive cells contributes to the assumption that CD44 acts as an early marker of differentiation[295]. CD73 and CD44 are both involved in lymphocyte signalling and they are both positive markers for MSCs, therefore these could be the reasons why they correlate with each other. CD73, originally defined as a lymphocyte differentiation antigen, is thought to function as a co-signalling molecule on T lymphocytes and as an adhesion molecule that is important for lymphocyte binding to endothelium[296,297]. The primary domains of CD44 are the extracellular domain, the transmembrane domain, and the intracellular domain. The transmembrane domain provides an avenue for interacting with co-factors and adaptor proteins as well as directing lymphocyte homing[298]. Furthermore, CD146 correlated with CD73 and CD44, therefore its levels will be higher when also these two markers are highly expressed. As mentioned above, CD146 is also expressed by lymphocytes and is involved in various physiological processes, including cell–cell and cell–matrix interactions, cell migration, and signalling. Therefore, it could be that these function similarities could be the reason why these markers correlate. ALP instead correlated negatively with CD73, allowing the assumption that this marker was not highly expressed when CD73 was present. Therefore, mesenchymal stem cells from the bone did not express much of this marker while they expressed CD73. Since both ALP and CD73 are involved in the metabolism of AMP to adenosine, it could be that they are expressed in a different way related to their role in the conversion from AMP to adenosine. The study of Jackson et al. suggested that when there are high ATP levels in the cells then CD73 is activated, while ALP is not, whereas when ATP levels are low, ALP is activated and CD73 is not[299]. The genomic stability of MSCs was subsequently tested for the three sampling groups. Genetic stability and potential transformation leading to tumour growth has become one of the most important safety concerns for stem cell therapies[158]. Chromosomal alterations are associated with increased tumourigenicity and the inability to reach desired differentiation states[160,165]. In this work, all the samples resulted as diploids and the three sampling methods did not seem to have an impact on their genomic content. In some samples, nuclei with an higher genomic content (≥ 9.5c) were detected and it was thought that it could be due to longer cultivation time, as it is already hypothesised in the literature[159]. However, the Spearman's correlation coefficient did not detect any significant association with the time of culture or with the storage method. Therefore, the results suggest that MSCs are affected by the sampling methods in their colony forming ability, proliferation and differentiation, however not in their marker expression and genomic

stability. The majority of the current literature also suggests that MSCs have usually stable karyotypes[166–169]. Subsequently these results were applied to create a quality score for the samples analysed. It is known that every MSC-donor is different (genetically, physiologically, etc.) and MSCs from different donors or sources are highly heterogenic, which is clearly one of the most important of the uncontrolled aspects of MSC-based therapies. Therefore, based on the here presented results, a new score, the MSC Quality Score, was created according to the cell's quality parameters in order to distinguish samples of good from poor quality. As mentioned above, MSCs are a very heterogenous population of cells; therefore, it could happen that cells from the same donor or from the same tissue of origin behave differently in culture, and thus it is important to find a way to characterize their quality parameters for future clinical applications. The MSC Quality Score was designed to classify the samples according to their quality parameters, such as CFU, proliferation assay, differentiation ability and FACS marker expression. From the results, it was possible to distinguish the three different sampling groups, having the fresh samples with the highest score, while the frozen bone samples had the lowest one, confirming the results from the cryopreservation study. According to the applied MSC Quality Score, a statistical difference regarding the cell quality could be detected ($p < 0.0313$). When then applying the MSC Quality Score also to other samples (data not shown) from other patients and tissues of origin (e.g., tibia proximal and femur distal), these samples had the same quality as the fresh samples from the proximal femur, confirming that the MSC Quality Score could be used also to analyse samples of different origin. Thus, the use of this score could have great applications in the clinic, since it could be introduced as an evaluation of the quality of MSCs, and therefore guide the decision which samples would be the most appropriate for therapy applications.

7 Conclusions & Future Perspectives

7.1 Pre-analytical Stability of Biomarkers

The temperature of storage had an impact on the biomarker levels overtime, which should not be underestimated. Furthermore, the "fresh" measurement should be introduced in biomarker studies as a new standard and reference for the analysis, to avoid a misinterpretation of the results. It represents the situation in the clinic and thanks to this reference it is possible to quantify the changes in the marker levels overtime. Careful planning should be applied to the collection and storage of biological samples to avoid compromising the diagnostic process. There are many potential sources of variability in biomarker quantification such as sample collection, processing and storage at the clinical site, which can compromise biomarker discovery analyses[220,221]. Validated biomarkers that can assess the quality of samples could be a useful tool for the evaluation of fit-for-purpose of any bio-specimen, and, when used routinely, should allow for proper inclusion or exclusion of a sample or results from that sample[300]. C3a desArg, CD26, M-CSF, S100A11 and M2PK have already been reported as tumour suppressors or activators in different types of cancer[301,229,302,303,247,244], therefore to know their behaviour during storage could be of great value for the diagnostic process and clinical trials.

7.2 Pre-analytic Impact for Clinical Biomarker Research

These results seem to confirm the impact of the temperature of storage on biomarker stability, as some markers would not have been detected as significant for pancreatic cancer when also storage at -80°C was considered. Furthermore, when considering only the samples stored in nitrogen, the AUCs and specificity and sensitivity of the single markers or of their combination further increased. In the logistic regression model, CD26 could be included only when using the samples stored in nitrogen. More studies should concentrate on the effects of long-term storage, since this information could have huge repercussions on the biomarker stability and therefore should be regarded as an important co-variable, like patient age and gender. Moreover, it could be interesting to analyse the markers used in this work also on different platforms, to verify if the results would remain the same, and therefore to be sure to not have false positives or kit/batch dependent results.

7.3 Mesenchymal Stromal Cells Quality Parameters Assessment

In conclusion, it is possible to isolate cells from frozen bone pieces and it is possible to cultivate them, as it is possible to cultivate isolated cells that were frozen. However, the sampling and storage method of MSCs have an impact on the proliferation rate, colony forming ability and differentiation ability. The sampling method had instead no effect on the genomic stability and marker expression. The results

confirmed that the sampling method has an impact on the properties of these cells and correlates with the time of culture, meaning that the frozen bone and the frozen cells will proliferate slower than the fresh condition. It could be hypothesized that the bone does not react well to freezing and that it would be more difficult to then extract vital cells from it. Nevertheless, it is also important to keep in mind that the incidence of MSCs in tissue is extremely low and seems to decrease with age[68]. Thus, it is also possible that the fraction of bone selected for the freezing did not contain a MSCs niche, or had a very low amount of them, which could explain the results. This is a risk that cannot be completely excluded in the evaluation of the results. It is also possible that this method should be optimised or that it could work for a shortened storage time, like overnight. Therefore, more data should be collected before giving a final statement on the applicability of this sampling method. The frozen cells presented instead a good colony forming ability and a good differentiation ability, being affected only in their proliferation rate by the storage. Thus, the method of the frozen cells should be preferred if it is not possible to directly isolate and cultivate the cells after tissue collection. More experiments should be performed to confirm these results, since the amount of samples used was not so high (24 samples, 8 per sampling condition) and this could have been the reason why there was no statistical significance among the groups. Since these samples should be then implemented for cell therapy, a medium composition without FBS that follows the directions of GMP-conformity would be advisable. Another possibility could be to improve the isolation method. It is important to distinguish between cryopreservation and sampling, since poorer results could have been caused by the sampling itself and not by the freezing. In this study, a gradient centrifugation followed by direct plating was performed, however some other methods could also be tried, for example to reduce the bone fat and collagen, which could together with the freezing compromise the quality of MSCs. To be able to collect the tissue and isolate the cells later on could have great advantages in helping the workflow in the clinic, therefore it is important to try to optimise this method. To find alternative approaches of sampling of MSCs and from different sources is crucial for therapy applications, since there are still many open questions about MSCs isolation and culture, as about their properties (e.g., proliferation, differentiation and markers expression), which are mostly caused by their heterogeneity. The importance of the quality parameters evaluation should also not be underestimated, since the characterising of the samples with the use of a score would enable researchers to know if the samples could be suited for therapy applications, allowing to overcome the challenge of MSCs heterogeneity.

8 References

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9 Appendix

9.1 Supplemental Tables & Figures

Supplemental Table 9.1 Assay ranges, intra- and inter-assay precision, recovery and sensitivity of each analyte used on the first version of the CRCSI and CRCSII chip. *This is the full measuring range after dilution

	CRCSI			**CRCSII**					
	CD26 ng/mL	**C3a desArg** ng/mL	**CRP** ng/mL	**CEA** ng/mL	**IL-8** pg/mL	**VEGF** pg/mL	**S100A11** ng/mL	**M-CSF** pg/mL	**NNMT** ng/mL
Full Assay Range	0 – 4,000*	0 – 120,000*	0 – 60,000 *	0 – 300	0 – 3,000	0 – 2,000	0 – 250	0 – 500	0 – 70
Precision – Intra-assay	≤10%	≤12%	≤8%	≤8%	≤10%	≤8%	≤9%	≤6%	≤10%
Precision – Inter-assay	≤9%	≤14%	≤8%	≤12%	≤8%	≤14%	≤14%	≤7%	≤12%
Recovery	≤95 - 103%	≤79 - 84%	≤94 - 103%	≤91 - 102%	≤92 - 98%	≤103 - 111%	≤90 - 99%	≤93 - 100%	≤79 - 90%
Sensitivity	26	0.58	0.998	0.39	6.95	4.48	2.91	2.61	0.77

Supplemental Table 9.2a Summary of clinical data of the pilot study group consisting of healthy control patients, colon adenoma patients, diverticulitis patients, colorectal cancer patients, colon cancer patients, liver metastasis patients, lung metastasis patients, pancreas cancer patients, pancreas adenoma patients, lung cancer patients, liver cancer patients, breast cancer patients, ovarian cancer patients and prostate cancer patients.

Parameter	Value	Healthy control patients (n=207)	Colon adenoma patients (n=142)	Diverticulitis patients (n=60)	Colorectal cancer patients (n=105)	Colon cancer patients (n=274)	Liver metastasis patients (n=25)	Lung metastasis patients (n=13)	Pancreas cancer patients (n=83)	Pancreas adenoma patients (n=15)	Lung cancer patients (n=15)	Stomach cancer patients (n=15)	Liver cancer patients (n=10)	Breast cancer patients (n=20)	Ovarian cancer patients (n=4)	Prostate cancer patients (n=10)
Gender	Female	116 (56%)	56 (39.4%)	32 (53.3%)	35 (33.3%)	109 (39.8%)	13 (52%)	3 (23%)	38 (45.8%)	8 (53.3%)	19 (38%)	6 (40%)	0	20	4	0
	Male	91 (44%)	86 (60.6%)	28 (46.7%)	70 (66.7%)	165 (60.2%)	12 (48%)	10 (77%)	45 (54.2%)	7 (46.7%)	31 (62%)	9 (60%)	10	0	0	10
Age (years)	Range	19 - 91	29 – 92	23 – 88	20 – 100	40 – 99	43 – 83	49 – 84	37 – 83	42 – 72	49 – 82	45 – 83	55 – 78	46 – 74	53 – 70	57 – 78
	Mean	61	66	62	68	70.7	66	67	64	59	69	69	69	62	64	69

Supplemental Table 9.2b Summary of clinical data of pancreas cancer patients of the pilot study group.

Parameter	Value	Pancreas cancer patients	%
UICC stage	1	4	4.8
	2	8	9.6
	3	42	50.6
	4	27	32.5
T status (tumour size)	1	4	4.8
	2	12	14.4
	3	36	43.3
	4	17	20.5
N status (nodal status)	0	13	15.6
	1	44	53
	2	1	1.2
	3	1	1.2
M status (distant metastasis)	0	18	21.7
	1	14	16.8
Tumour grading	G1	12	14.4
	G2	13	15.6
	G3	25	30.1

Supplemental Table 9.3a Summary of clinical data of the subset cohort of the pilot study group consisting of healthy control patients and pancreas cancer patients.

Parameter	Value	Healthy volunteers (n=168)	Pancreas cancer patients (n=74)
Gender	Female	95 (56.5%)	33 (44.6%)
	Male	73 (43.5%)	41 (55.4%)
Age (years)	Range	20 – 91	37 – 83
	Mean	60.7	64

Supplemental Table 9.3b Summary of clinical data of the pancreas cancer patients of the subset cohort of the pilot study group.

Parameter	Value	Pancreas cancer patients	%
UICC stage	1	4	5.4
	2	8	10.8
	3	36	48.6
	4	24	32.4
T status (tumour size)	1	4	5.4
	2	10	13.5
	3	32	43.2
	4	16	21.6
N status (nodal status)	0	13	17.5
	1	38	51.3
	2	1	1.3
	3	1	1.3
M status (distant metastasis)	0	13	17.5
	1	12	16.2
Tumour grading	G1/G2	23	31
	G3	24	32.4

Supplemental Table 9.4 Assay ranges, recovery and sensitivity of each analyte used on the M2PK and IL-18 ELISA. *This is the full measuring range after dilution

	M2PK ng/mL	**IL-18** pg/mL
Full Assay Range	0 – 128*	0 – 2,500*
Recovery	73 – 77 %	77 – 84 %
Sensitivity	3.4	42.8

Supplemental Table 9.5 Comparison between median serum levels of the six biomarkers after 1 year of storage at different ultralow temperatures

	C3a desArg ng/ml	**CD26 ng/ml**	**M-CSF pg/ml**	**S100A11 ng/ml**	**M2PK ng/ml**	**IL-18 pg/ml**
-80°C	499.4	638.3	4.417	1.31	16.64	0.0
-170°C	387.1	614.6	4.239	1.365	14.15	0.0
p-value	<0.001	0.597	0.271	0.272	0.034	0.304
-80°C	499.4	638.3	4.417	1.31	16.64	0.0
-80°C/-170°C	443.3	633.9	4.238	1.377	15.82	0.0
p-value	0.019	0.650	0.149	0.241	0.384	0.256
-80°C/-170°C	443.3	633.9	4.238	1.377	15.82	0.0
-170°C	387.1	614.6	4.239	1.365	14.15	0.0
p-value	<0.001	0.705	0.705	0.970	0.120	0.942

Supplemental Table 9.6 Spearman´s Correlation coefficient above 0.4 (all with p<0.001) for the stability cohort study

	C3a des Arg	**CD26**	**M-CSF**	**S100A11**	**M2PK**	**IL-18**
C3a des Arg	n.a.			0.424		
CD26		n.a.				
M-CSF			n.a.			0.545
S100A11	0.424			n.a.		-0.494
M2PK					n.a.	
IL-18			0.545	-0.494		n.a.

Supplemental Table 9.7a Summary of clinical data of the samples stored below -130°C consisting of healthy control patients and pancreas cancer patients. *for 1 pancreas cancer patient no information about the gender

Parameter	Value	Healthy control patients (n=37)	Pancreas cancer patients (n=135)*
Gender	Female	18 (48.7%)	65 (48.1%)
	Male	19 (51.3%)	69 (51.1%)
Age (years)	Range	33 – 84	38 – 84
	Mean	59.7	65.7

Supplemental Table 9.7b Summary of clinical data of the pancreas cancer patients stored below -130°C.

Parameter	Value	Pancreas cancer patients	%
UICC stage	1	6	4.4
	2	19	14
	3	67	49.6
	4	13	9.6
T status (tumour size)	1	3	2.2
	2	9	6.6
	3	84	62.2
	4	6	4.4
N status (nodal status)	0	25	18.5
	1	74	54.8
	2	1	0.7
	3	1	0.7
M status (distant metastasis)	0	9	6.6
	1	9	6.6
Tumour grading	G1	9	6.6
	G2	54	40
	G3	32	23.7

Supplemental Table 9.8 Spearman´s Correlation coefficient above 0.4 (moderate correlation) (all with p<0.001) (only N_2 samples considered)

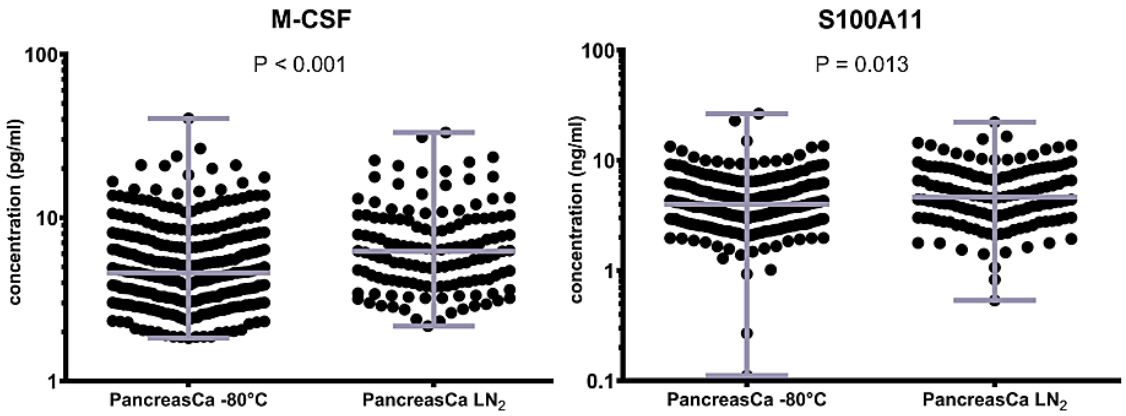
	G	T	N	M	Gender	Age	C3a des Arg	CD26	M-CSF	S100A11	Cyfra	CEA	CA19-9
G	n.a.												0.542
T		n.a.											
N			n.a.										
M				n.a.									
Gender					n.a.								
Age						n.a.							
C3a des Arg							n.a.			0.462			
CD26								n.a.					
M-CSF									n.a.	0.422			
S100A11							0.462		0.422	n.a.			
Cyfra											n.a.		0.595
CEA												n.a.	
CA19-9	0.542										0.595		n.a.

Supplemental Figure 9.1 a) Median serum levels of pancreatic cancer samples for the four biochip markers and the three routine markers at -80°C and <-130°C storage. M-CSF and S100A11 showed a statistical difference when stored in these two conditions; **b)** Dot plots for M-CSF and S100A11 in pancreatic cancer samples, which show a statistical difference between -80°C and nitrogen storage. The results are represented in a logarithmic scale.

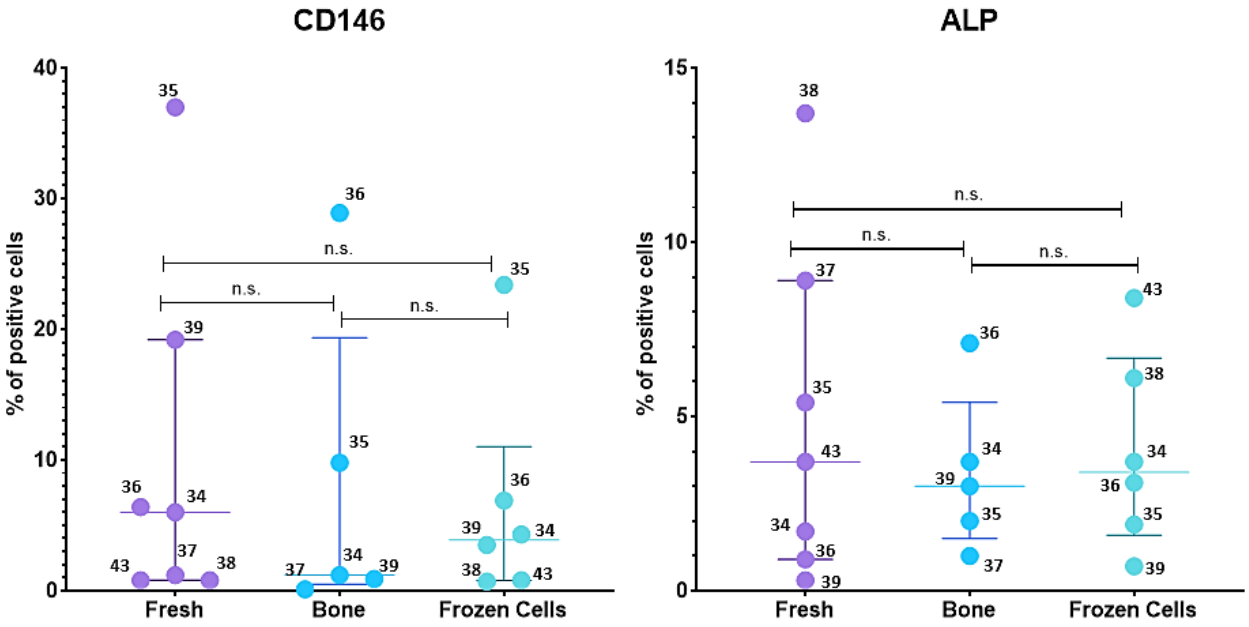
a

	C3a desArg (ng/ml)	CD26 (ng/ml)	M-CSF (pg/ml)	S100A11 (ng/ml)	Cyfra (ng/ml)	CEA (ng/ml)	CA19-9 (U/ml)
-80°C Median	1140.4	524.9	4.59	3.99	2.7	3	186
LN_2 Median	1458.6	541.4	6.13	4.64	2.95	2.85	187.5
P-value	0.145	0.067	< 0.001	0.013	0.701	0.575	0.529

b



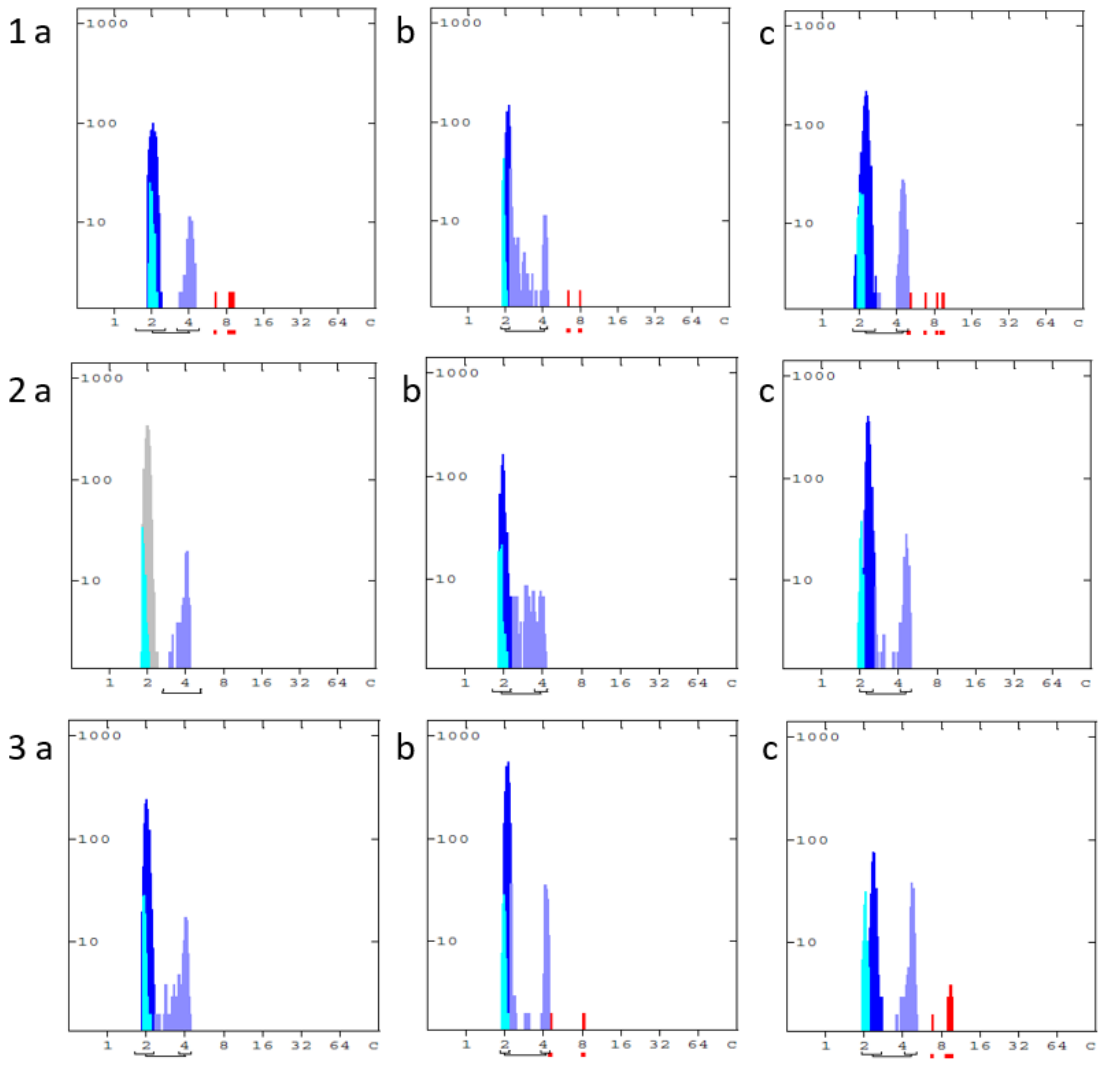
Supplemental Figure 9.2. Box plot of CD146 and ALP results of the FACS analysis among "fresh" cells (purple), cells isolated from the frozen bone (blue) and isolated and subsequently cryopreserved cells (green). The percentage of positive cells for these two markers is heterogeneous among the patients and among the three difference sampling conditions. No statistical difference was detected among the three sampling groups for the four markers.



Supplemental Table 9.9 Spearman´s Correlation coefficient above 0.4 (moderate correlation) (all with $p < 0.05$).

	Storage condition	Time of culture	Age	Gender	CFU	Proliferation	Alkaline phosphatase activity	Osteogenic diff	Adipogenic diff	CD14	CD34	CD146	ALP	CD73	CD44
Storage condition	**n.a.**	.585				-.649					-.478				
Time of culture	.585	**n.a.**				-.604					-.565			-.624	-.666
Age			**n.a.**							.475		.634			
Gender				**n.a.**										.499	
CFU					**n.a.**	.566	.417								
Proliferation	-.649	-.604			.566	**n.a.**			.450	-.470	.525				
Alkaline phosphatase activity					.417		**n.a.**	.477						.618	
Osteogenic diff							.477	**n.a.**							
Adipogenic diff						.450			**n.a.**						
CD14			.475			-.470				**n.a.**					
CD34	-.478	-.565				.525					**n.a.**				.452
CD146			.634									**n.a.**		.555	.505
ALP													**n.a.**	-.583	
CD73		-.624		.499			.618					.555	-.583	**n.a.**	.518
CD44		-.666									.452	.505		.518	**n.a.**

Supplemental Figure 9.3 Examples of ploidy measurements on three different patients, for the three sampling conditions. In the first patient (1), the nuclei of MSCs were diploids, however some nuclei with a higher genomic content were identified in the a) fresh condition, b) frozen cells condition and c) frozen bone condition. In the second patient (2), all the cells were diploids in the three conditions, while in the third patient (3), a) the fresh condition was diploid, and some cells with an aneuploid nuclei content appeared in the b) frozen cells condition and in the c) frozen bone condition.



9.2 Abbreviations

ADP	adenosine diphosphate
AJCC	American Joint Committee on Cancer
AKT	RAC-alpha serine/threonine-protein kinase
ALP	alkaline phosphatase
AMP	adenosine monophosphate
APC	allophycocyanin
AR-S	Alizarin Red stain
ARID1A	AT-rich interactive domain-containing protein 1A
ASP	acylation stimulating protein
ATP	adenosine triphosphate
AUC	area under the curve
BD-IPMN	branch duct- intraductal papillary mucinous neoplasm
BM	bone marrow
BMAC	bone-marrow aspiration concentrate
BMSC	Bone marrow mesenchymal stem cells
BRISQ	Bio-specimen Reporting for Improved Study Quality recommendations
BRL	rosiglitazone
BSA	bovine serum albumine
C3a desArg	complement component 3a
CA19-9	carbohydrate antigen 19-9
CAFs	carcinoma associated fibroblasts
CAPS	International Cancer of the Pancreas Screening
CCD	charged coupled device
CD	cluster of designation
CD14	cluster of differentiation 14 protein
CD26	protein 26
CD34	transmembrane phosphoglycoprotein protein
CD44	homing cell adhesion molecule
CD73	Ectoenzymes ecto-5’-nucleotidase
CD146	cell surface glycoprotein MUC18

CDKN2A	cyclin-dependent kinase Inhibitor 2A
CEA	carcinoembryonic antigen
CFU	colony-forming unit
CFU-F	colony-forming unit-fibroblasts
CPA	cryoprotective agent
CPC	cetylpyridinium chloride
CRP	C-reactive protein
CT	computed tomography
Cyfra	cytokeratin 19 fragment
DAPI	4’,6-diamidino-2-phenylindole
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
ESCs	embryonic stem cells
EUS	endoscopic ultrasonography
FACS	fluorescence-activated cell sorting
FAMMM	familial atypical multiple mole and melanoma syndrome
FAP	familial adenomatous polyposis syndrome
FBS	foetal bovine serum
FBXW7	F-box/WD repeat-containing protein 7
FITC	fluorescein isothiocyanate
FSC	forward scatter
GAP	GTPase-activating proteins
GMP	Good Manufacturing Practice
GNAS	GNAS complex locus
GvHD	graft-versus-host disease
GTP	Guanosine-5'-triphosphate
hBM-MSC	human bone marrow-derived mesenchymal stromal (stem) cells
HBOC	hereditary breast and ovarian cancer syndrome

HCl	Hydrochloric acid
hES	human embryonic stem cells
HNPCC	hereditary nonpolyposis colorectal cancer
HP	hereditary pancreatitis
ICB-L	*Interdisciplinary Centrum for Biobanking-Lübeck*
IBMX	3-Isobutyl-1-methylxanthine
IL-8	interleukin 8
IL-18	interleukin-18
IPMN	intraductal papillary mucinous neoplasm
iPSC	induced pluripotent stem cells
ISBER	International Society for Biological and Environmental Repositories
ISO	International Organisation for Standardisation
ISCT	International Society for Cellular Therapy
ITPN	intratubular papillary neoplasm
KRAS	Kirsten rat sarcoma viral oncogene homolog
LN2	liquid nitrogen
M1	pro-inflammatory or ‘classically activated’ macrophages
M2	anti-inflammatory or ‘alternatively activated’ macrophages
M-CSF	macrophage colony-stimulating factor
M2PK	M2 pyruvate kinase
MCN	mucinous cystic neoplasm
MD-IPMN	main duct- intraductal papillary mucinous neoplasm
MDSC	myeloid-derived suppressor cell
MEM	Minimum Essential Medium
MgCl2	magnesium chloride
MIC1	macrophage inhibitory cytokine 1
MLL3	myeloid/lymphoid or mixed-lineage leukemia protein 3
MMP7	matrix metalloproteinase-7
MNC	mononuclear cells
MRCP	magnetic resonance cholangiopancreatography
MRI	magnetic resonance imaging

MSC	mesenchymal stromal (stem) cells
MSLN	mesothelin
N_2	Nitrogen
Na2S2O5	sodium metabisulfite
NaHCO3	sodium bicarbonate
NaOH	sodium hydroxid
NCI	National Cancer Institute
NIH	National Institutes of Health
NNMT	nicotinamide N-methyltransferase
OPN	osteopontin
p16INK4A	cyclin-dependent kinase inhibitor 2A
p/s	penicillin-streptomycin
PanIN	pancreatic intraepithelial neoplasm
PBS	phosphate-buffered saline
PDAC	pancreatic ductal adenocarcinoma
PE	phycoerythrin
PEP	phosphoenolpyruvate
PFA	paraformaldehyde
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PJ	Peutz-Jeghers Syndrome
QCs	quality controls
RNF43	ring finger protein 43
ROC	receiver operator characteristic
S100A11	S100 calcium-binding protein A11
SMAD2	mothers against decapentaplegic homolog 2
SMAD3	mothers against decapentaplegic homolog 3
SOP	standard operation procedures
SPREC	Standard PRE-analytical Code
SSC	side scatter
TBS	Trizma® base
Tg	glass transition temperature of water

TGFβ	transforming growth factor beta
TIMP-1	tissue inhibitor of metalloproteinase-1
TLRs	Toll-like receptors
TNM	classification of malignant tumours (T: tumor stage, N: lymph node stage; M: metastasis stage)
TP53	tumour protein 53
UICC	International Union for Cancer Control
VEGF	vascular endothelial growth factor
WHO	World Health Organization

9.3 List of Figures

Appendix

9.4 List of Tables

Supplemental Table 9.6	Spearman´s Correlation coefficient above 0.4 (all with $p<0.001$) for the stability cohort study.
Supplemental Table 9.7a	Summary of clinical data of the samples stored below -130°C consisting of healthy control patients and pancreas cancer patients.
Supplemental Table 9.7b	Summary of clinical data of the pancreas cancer patients stored below -130°C.
Supplemental Table 9.8	Spearman´s Correlation coefficient above 0.4 (moderate correlation) (all with $p < 0.001$) (only N_2 samples considered).
Supplemental Table 9.9	Spearman´s Correlation coefficient above 0.4 (moderate correlation) (all with $p < 0.05$).

10 Publication List

10.1 Articles as part of this thesis

Giulia Facchinetti, Timo Gemoll, Katja Klempt-Giessing, Philip Lowry, and Jens K Habermann. Temperature and storage time influence the stability of cancer biomarkers in human serum. *Manuscript prepared for submission.*

Giulia Facchinetti, Katja Klempt-Giessing, Sandra Freitag-Wolf, Stefan Holdenrieder, Michael Linnebacher, Katarina Cuk, Hermann Brenner, Cenap Güngör, Christian Röder, Emily P Slater, Philip Lowry, Timo Gemoll, and Jens K Habermann. Clinical performance of a multiplex biochip for the detection of pancreatic cancer is affected by the temperature of storage. *Manuscript prepared for submission.*

Giulia Facchinetti, Lina Nießen, Timo Gemoll, Arndt P Schulz and Jens K Habermann. The impact of cryopreservation on femoral bone derived mesenchymal stromal (stem) cells. *Manuscript prepared for submission.*

10.2 Articles not included in this thesis

Thorben Sauer, **Giulia Facchinetti**, Michael Kohl, Justyna M. Kowal, Svitlana Rozanova, Julia Horn, Hagen Schmal, Ivo Kwee, Arndt-Peter Schulz, Sonja Hartwig, Moustapha Kassem, Jens K. Habermann and Timo Gemoll. Protein Expression of AEBP1, MCM4, and FABP4 Differentiate Osteogenic, Adipogenic, and Mesenchymal Stromal Stem Cells. *Published, Int J Mol Sci. 2022 Feb 25;23(5):2568.*

Lina Nießen, **Giulia Facchinetti**, Till Elsner, Robert Wendlandt, Timo Gemoll, Jens K. Habermann and Arndt-Peter Schulz. Effect of volume flows on the viability of MSCs during injection through a cannula. *Published, De Gruyter, Current Directions in Biomedical Engineering 2021;7(2): 272-275.*

11 Scientific Contributions

11.1 Scientific Talks

2016

Giulia Facchinetti, Timo Gemoll, Ina Gursinski, Philip Lowry, Alex Chacko, Jens K Habermann. Stability test of a novel multiplex-protein array for serum diagnostics of pancreatic cancer: impact of temperature and time of storage.

20. Chirurgische Forschungstage, Magdeburg, 08. – 10. September 2016

Abstract published in: Eur Surg Res 2016;57:263–335 (Abstract ID: 128).

11.2 Poster Presentations

2019

Giulia Facchinetti, Lina Niessen, Timo Gemoll, Regina Maushagen, Martina Oberlaender, Lena Figge, Ann-Kristin Kock-Schoppenhauer, Petra Duhm-Harbeck, Andreas Unger, Klaus Waizner, Robert Wendlandt, Wiebke Zweig, Silke Burdinski, Justyna M. Kowal, Anders Haakonsson, Christina Bober, Kai Diercks, Roman Nassutt, Robin Buescher, Ralf Schwanbeck, Nils Reimers, Hagen Schmal, Ralf Duckert, Ines Kröger, Gesine Stueck, Stefanie Kessel, Mirjam Hecht, Thomas Frahm, Anna Eckers, Julia Brilling, Torben Barington, Moustapha Kassem, Arndt-Peter Schulz, Jens K. Habermann. Influence of cryopreservation on bone cells quality parameters.

Europe Biobank Week, Lübeck, 09. – 11. October 2019

Giulia Facchinetti, Katja Klempt-Giessing, Sandra Freitag-Wolf, Stefan Holdenrieder, Michael Linnebacher, Katarina Cuk, Hermann Brenner, Cenap Güngör, Christian Röder, Emily P Slater, Detlef K Bartsch, Philip Lowry, Timo Gemoll, and Jens K Habermann. Protein biochip assessment of pre-analytical impact of storage on serum samples.

Europe Biobank Week, Lübeck, 09. – 11. October 2019

Giulia Facchinetti, Lina Niessen, Timo Gemoll, Regina Maushagen, Martina Oberlaender, Lena Figge, Ann-Kristin Kock-Schoppenhauer, Petra Duhm-Harbeck, Andreas Unger, Klaus Waizner, Robert Wendlandt, Wiebke Zweig, Silke Burdinski, Justyna M. Kowal, Anders Haakonsson, Christina Bober, Kai Diercks, Roman Nassutt, Robin Buescher, Ralf Schwanbeck, Nils Reimers, Hagen Schmal, Ralf Duckert, Ines Kröger, Gesine Stueck, Stefanie Kessel, Mirjam Hecht, Thomas Frahm, Anna Eckers, Julia Brilling, Torben Barington, Moustapha Kassem, Arndt-Peter Schulz, Jens K. Habermann. The impact of cryopreservation on femoral bone derived mesenchymal stromal (stem) cells.

10. Symposium für industrielle Zelltechnik, Lübeck, 05. – 06. September 2019

2017

Giulia Facchinetti, Timo Gemoll, Stefanie Arndt, Katja Klempt-Giessing, Philip Lowry, Jens K Habermann. The effect of temperature and time-of-storage on the stability of biomarkers for serum based diagnostics of pancreatic cancer.
Global Biobank Week, Stockholm, 13. – 15. September 2017

Timo Gemoll, **Giulia Facchinetti**, Friederike Praus and Jens K Habermann. Sample quality considering tumour heterogeneity: Optimized workflow for top-down protein isolation from solid tumours in clinical research.
Global Biobank Week, Stockholm, 13. – 15. September 2017

Giulia Facchinetti, Timo Gemoll, Stefanie Arndt, Katja Klempt-Giessing, Philip Lowry, Jens K Habermann. Impact of temperature and time-of-storage on the stability of biomarkers for a novel multiplex-protein array based serum diagnostics of pancreatic cancer.
Proteomic Forum 2017, Potsdam, 02. – 05. April 2017

2016

Giulia Facchinetti, Timo Gemoll, Stefanie Arndt, Katja Klempt-Giessing, Philip Lowry, Alex Chacko, Jens K Habermann. The impact of pre-analytical conditions: Investigation of a novel multiplex-protein array for serum diagnostics of pancreatic cancer.
198. Tagung der Vereinigung Norddeutscher Chirurgen, Hamburg, 25. – 26. November 2016

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