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Analysis of circulating markers in patients with solid tumours

Analýza cirkulujících markerů u pacientů se solidními nádory

MSc. thesis

Supervisor: doc. RNDr. Pavel Souček, CSc.

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Prehlásenie:

Prehlasujem, že som záverečnú prácu spracovala samostatne a že som uviedla všetky použité informačné zdroje a literatúru. Táto práca ani jej podstatná časť nebola predložená k získaniu iného alebo rovnakého akademického titulu.

V Prahe, 12.8.2019

Podpis

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Abstrakt:

Cirkulujúca cell-free DNA (cfDNA) a jej frakcia, cirkulujúca tumorová DNA, pochádzajúca z nádoru, sú považované za inovatívny prognostický a prediktívny biomarker vo svete onkologickej diagnostiky. Mnohé štúdie preukázali pozmenené hladiny koncentrácie cfDNA a integrity - indikátora množstva ctDNA v rámci cfDNA, v telových tekutinách u pacientov s nádorovými ochoreniami v porovnaní so zdravými jedincami, čo poukazuje na ich potenciál ako efektívneho biomarkeru na monitorovanie dynamiky týchto ochorení. Táto práca sa sústredí na optimalizáciu a validáciu kvantifikačných metód, ktoré sú následne použité na analýzu spomínaných parametrov cfDNA u vzoriek štyroch rôznych nádorov. Najskôr boli otestované dva rôzne komerčné kity na izoláciu cfDNA u vzoriek plazmy a séra. Metódy na kvantifikáciu, kvantitatívna real-time polymerázová reakcia (qPCR) a PicoGreen dsDNA assay, boli optimalizované na efektívne kvantifikovanie nízkych koncentrácií cfDNA, a následne porovnané medzi sebou a ku droplet digital PCR, ktorá bola použitá na vybraný počet vzoriek. Zároveň bola stanová koncentrácia a integrita cfDNA vzoriek karcinómu prsu, vaječníkov, kolorekta a pankreasu. Väčší výťažok cfDNA bol získaný pomocou kitu QIAamp Circulating Nucleic Acid isolation kit (Qiagen) v porovnaní s kitom Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit (Norgen). Koncentrácia cfDNA všetkých menovaných ochorení bola zvýšená v porovnaní so zdravými kontrolami. Hodnoty cfDNA integrity sa na druhej strane správali odlišne. Zatiaľ čo sa cfDNA integrita u karcinómu prsníka a pankreasu nelíšila od zdravých kontrol, u pacientov s karcinómom kolorekta a vaječníkov bola v porovnaní so zdravými jedincami znížená. Tieto výsledky podporujú hypotézu, že cfDNA pochádzajúca z nádoru je viac fragmentovaná ako cfDNA zo zdravých buniek a dokazujú, že cfDNA je vhodným kandidátom pre detailnejšie štúdium zaoberajúce sa ich dynamikou u pacientov s nádorovými ochoreniami.

Kľúčové slová: cirkulujúce biomarkery, cell-free DNA, DNA integrita, DNA kvantifikačné metódy, optimalizácia

Abstract:

Circulating cell-free DNA (cfDNA) and its tumour-derived circulating tumour DNA (ctDNA) fraction are considered an innovative prognostic and predictive biomarker in oncological diagnostics. Many studies have demonstrated higher levels of cfDNA concentration and integrity, as an indicator of the amount of ctDNA in cfDNA, in body fluids from patients with cancer diseases in comparison with healthy individuals, which suggest its potential as an effective biomarker for monitoring of the tumour dynamics. This study focused on optimisation and validation of measurement methods later used for analysis of cfDNA concentration and integrity in blood samples from patients with four different solid cancers. Two different commercial isolation kits have been tested in plasma and serum samples. Quantitative real-time polymerase reaction (qPCR) and PicoGreen dsDNA assay were optimized to effectively quantify low concentrations of cfDNA, subsequently compared to each other and to droplet digital PCR assay tested on selected samples. The concentration and integrity of cfDNA from plasma samples of breast, ovarian, colorectal and pancreatic cancer patients were evaluated. Higher amounts of cfDNA were obtained by the QIAamp Circulating Nucleic Acid isolation kit (Qiagen) in comparison to Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit (Norgen). The cfDNA levels in plasma samples from patients with mentioned diseases were higher compared to plasma samples obtained from healthy individuals. On the other hand, cfDNA integrity behaved differently. While cfDNA integrity in plasma samples of breast and pancreatic carcinoma patients did not differ from controls, the same parameter was lower in patients with colorectal and ovarian carcinoma in comparison to healthy individuals. These results support the hypothesis, that cfDNA which originates in tumour may be more fragmented compared to cfDNA from healthy cells and prove that cfDNA is a good candidate for detailed study of its dynamics in patients with solid tumours.

Key words: circulating biomarkers, cell-free DNA, DNA integrity, DNA quantification, optimisation

Contents:

Abbreviations

ACTB	beta (β)-actin
BC	breast cancer
BRAF	serine/threonine-protein kinase B-Raf
CA15-3	carcinoma antigen 15-3
CA19-9	carbohydrate antigen 19-9
CEA	carcinoembryonic antigen
cfDI	cell-free DNA integrity index
cfDNA	cell-free DNA
cfRNA	cell-free RNA
CRC	colorectal cancer
CTC	circulating tumour cell
ctDNA	circulating tumour DNA
ddPCR	droplet-digital polymerase chain reaction
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ER	oestrogen receptor
FDA	The Food and Drug Administration
HCC	hepatocellular cancer
HER2	human epidermal growth factor receptor 2
KRAS	Kirsten rat sarcoma viral oncogene
LINE	long interspersed nuclear elements
LOH	loss of heterozygosity
MAPK	mitogen-activated protein kinase
MET	mesenchymal-epithelial transition
miRNA	micro-RNA
MSI	microsatellite instability
NGS	next-generation sequencing
NPC	nasopharyngeal cancer
NSCLC	non-small cell lung cancer
NTC	no-template control
OvC	ovarian cancer
PDAC	pancreatic ductal adenocarcinoma
PIK3CA	phosphoinositide-3-kinase, catalytic, α polypeptide
PR	progesterone receptor
PSA	prostate-specific antigen

qPCR	quantitative real-time polymerase chain reaction
SEPT9	septin9
Tis	tumour in situ
TP53	tumour protein 53

1 Introduction

Oncological diseases are responsible for millions of new cases and deaths every year. One of the greatest obstacles in cancer diagnostics remains insufficient screening programs for early diagnosis and often inaccurate prognostic and predictive biomarkers, which may lead to the selection of inappropriate treatment or underestimating of tumour aggressiveness and recurrence. The need for new, fast and cheap biomarkers is rising.

Cell-free DNA (cfDNA) is a promising innovative approach possessing features of a prognostic and predictive biomarker for carcinoma patients. The fact that fraction of the cfDNA originates from tumour cells provides insight into the character of the particular tumour, and its fluctuant levels can reflect the real-time status of the tumour burden. Additionally, cfDNA fragment length (integrity) may also serve as a marker, following the hypothesis that long circulating DNA fragments originate from necrotic tumour cells, whereas short fragments are derived from apoptotic cells. However, differences in measurement methods and their insensitivity to small amounts of cfDNA often represent a limitation of this biomarker.

Because of these limitations, the first aim of this thesis was optimisation and validation of appropriate methods to quantify cfDNA and evaluate the cfDNA integrity index (cfDI) in blood samples. Another part of this study consists of sample measurement from patients of breast, ovarian, colorectal and pancreatic cancer. The result of this thesis provides validated methods for the measurement of these cfDNA parameters and eventually suggesting their future improvements. The findings indicate changing and dynamic levels of cfDNA concentration and cfDI in the blood of patients according to their carcinoma type and stage and potentially provide a ground for further research of this field.

2 Literature overview

2.1. Epidemiology of cancer

According to the World health organisation, cancer is the second leading cause of death worldwide. In 2018 was estimated 18.1 million of new cases and approximately 9.6 million deaths worldwide (fig.1, GLOBOCAN 2018). In many cases, cancer remains unnoticed until advanced stages, as the progress of the disease may be asymptomatic. Therefore, the routine and preventive examination could prevent many fatal consequences.

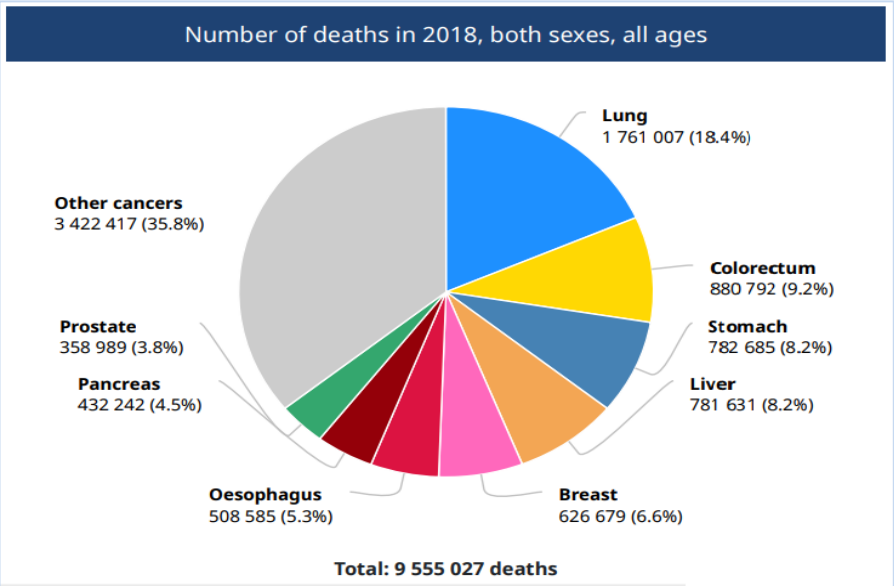

Figure 1. Number of deaths worldwide caused by cancer (GLOBOCAN 2018).

In recent years, improvement in technology and the rise of screening programs have shown an interesting trend in incidence in some types of cancers, as can be seen on the example of prostate and breast cancer incidence in the Czech Republic. The increasing incidence is observable in both sexes throughout years, whereas mortality shows smaller change (fig.2, ÚZIS ČR). The presumable explanation is routine examination of prostatic specific antigen (PSA), a biomarker used in diagnostics of prostate cancer. In the case of breast cancer, preventive National breast cancer screening program has started in the year 2002 in the Czech Republic, explaining rising incidence of breast oncological disease in women, but also successful capture of early-stages of breast cancer. However, breast cancer remains one of the leading oncological causes of death in women in the Czech Republic (Cancer incidence, ÚZIS ČR).

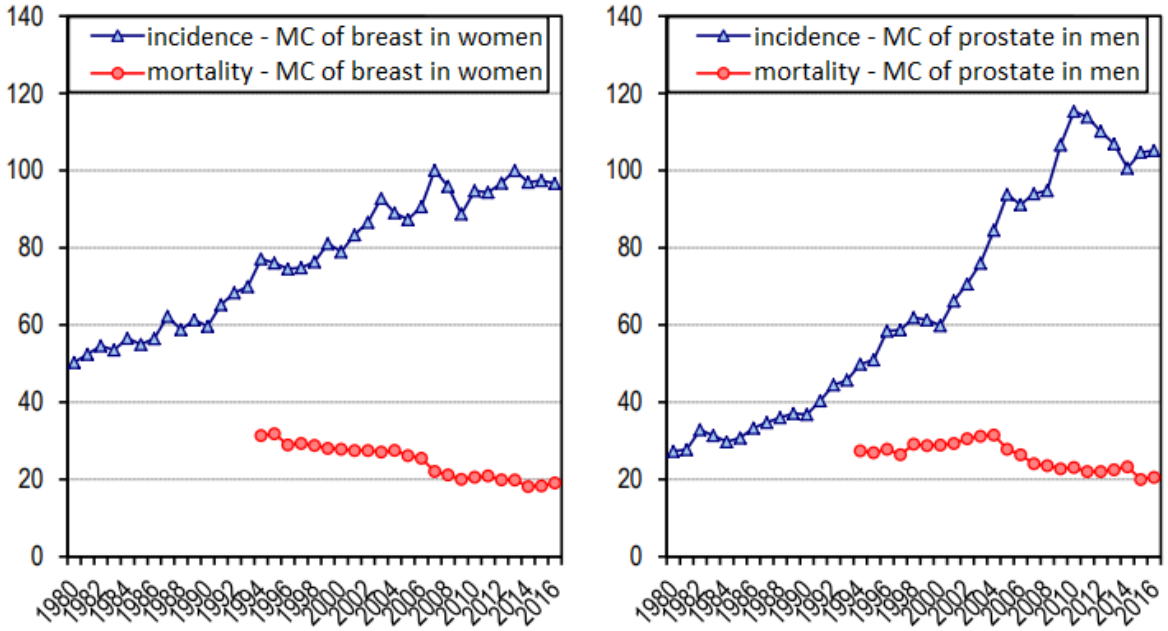

Figure 2. Trend of incidence and mortality of malignant prostate cancer in males and malignant breast cancer in females. Statistical data from year 1980 to 2016 in Czech Republic.
MC = malignant cancer (Adapted from ÚZIS ČR, world age-standardised rates).

Another common oncological disease in the Czech Republic is colorectal cancer, with 7610 cases in 2016. In recent years, the incidence in both sexes slightly decreased, probably again thank to initiation of National screening program from the year 2000 for individuals over 50 years and better general awareness of the disease and life style (Cancer incidence 2016, ÚZIS ČR; Zavoral et al., 2011).

Table 1. Incidence and mortality of selected solid tumour in the Czech Republic in 2016

	incidence	*mortality*	
absolute	7869	1921	*breast cancer (women)*
per 100 000	146.47	35.76	
absolute	998	628	*ovarian cancer (women)*
per 100 000	18.58	11.69	
absolute	7610	3746	*colorectal cancer*
per 100 000	73.79	35.46	
absolute	2243	1982	*pancreatic cancer*
per 100 000	21.23	18.76	

(ÚZIS ČR)

The importance of preventive screening, its accuracy and reliability can be also seen from the example of incidence and mortality of pancreatic cancer, which is very hard to diagnose in less advanced/early stages. Czech Republic had the eighth highest rate of pancreatic cancer incidence in 2018 (GLOBOCAN database). Unfortunately, the most of the cases are diagnosed in the advanced stages, where the therapy is virtually ineffective (fig. 3).

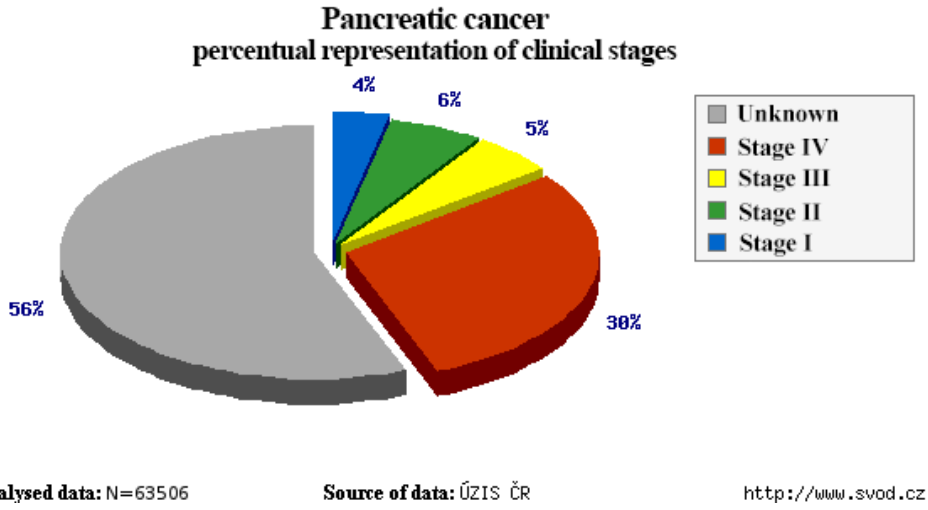

Figure 3. Incidence of individual stages of pancreatic cancer. Clinical data are established based on TNM classification valid at the time of diagnosis (Adapted from ÚZIS ČR).

2.2. TNM classification

TNM classification is internationally accepted standard approach for cancer staging. At present, TNM classification is determined according to the 8th Edition of the UICC TNM classification of Malignant Tumors, Czech version was published in 2017. Category T describes tumour size, category N describes whether lymph node metastases are present, and category M describes presence of distant metastases. Classification by stage is probably the most important for the determination of prognosis and treatment. TNM parameters are used for classification of tumour into stage groups, from Stage 0 to Stage IV. Every cancer diagnosis has specific criteria for staging. Pathological TNM classification (pTNM) is evaluated from resected tumour and nodal tissues during surgical treatment and clarifies clinical TNM classification (cTNM), obtained by imaging methods, which is important for an appropriate treatment selection (Sobin et al., 2011).

This study focuses on four different diseases – breast, ovarian, colorectal and pancreatic cancer. According to the eight edition of TNM classification, the staging of the four diseases is carried out similarly, except few differences. In breast, colorectal and

pancreatic cancer, 4 levels (T1-4) are distinguished in T category according to the size of tumour tissue and type of tissue into which has tumour expanded. In the case of ovarian cancer, category T consists of only three categories T1-T3, which have another three subcategories a-c, describing events such as whether single or both ovaries are affected, whether and where are malignant cells or microscopic metastases present, etc. N category for ovarian cancer describes if the metastases are or are not present in regional lymphatic nodules (N0 or 1), whilst in breast, colorectal and pancreatic cancer several subgroups exist. Subcategories N1 and N2 in pancreatic and N1a-c with N2a-b in colorectal disease define mainly a number of affected regional nodules. In breast cancer, subcategories N1, N2a-b and N3a-c characterise not only amount but also specific location of metastases in the nodules. N1a-b represents the size of regional lymph node metastases in pancreatic cancer. M category is very similar for all the diseases and can be complemented with specific location of distant metastases, or if one or more organs are affected (Sobin et al., 2011; Brieley et al., 2017). TNM staging of the samples utilised in this study was conducted according to TNM valid at the time of diagnosis.

Besides TNM classification, clinical information usually involves additional important characteristics about the particular tumour, as tumour grade, angioinvasion, or expression of protein markers. These data are specific for each tumour type and are also necessary for the establishment of best available therapy. In order to obtain such data, clinical examinations are required. One of the first steps of the diagnostic process is characterisation of the tumour using imaging methods such as computed tomography, positron emission tomography scan and magnetic resonance, and analysis of tumour markers from patient's blood and tissue of the tumour.

2.3. Tumour markers

Detection of tumour markers is an essential part not only of diagnosing but also of monitoring of the disease's dynamics. Tumour markers can be genes, transcripts or proteins with modified sequence and consequently with altered structure, expression, amount or function, suggesting abnormal cell behaviour (fig.4). They can be detected in solid tumour tissue (primary tumour tissue or metastases), or from body fluid such as serum or plasma, urine, stool or cerebrospinal fluid (Lindblom and Liljegren, 2000).

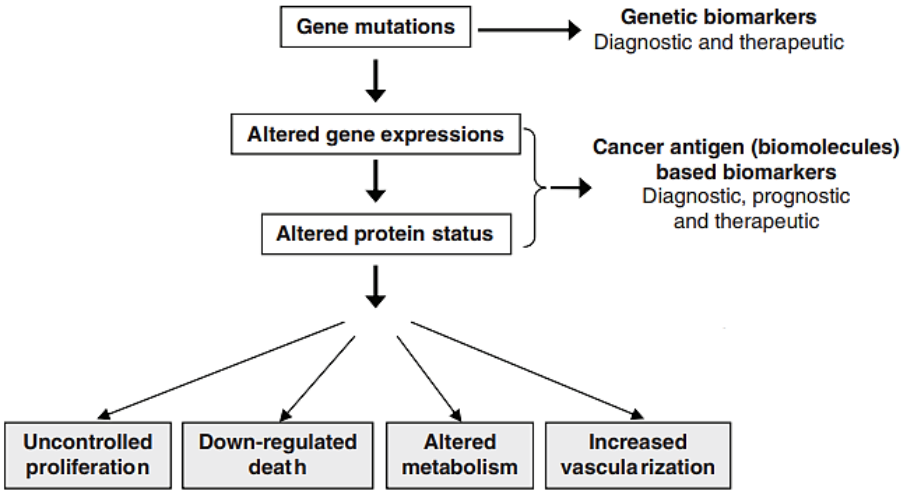

Figure 4. Pathways of tumorigenesis provide opportunities for identification of biomarkers.
(Edited and adapted from Bhatt et al., 2010)

An ideal tumour marker should have high sensitivity, or positive predictive value, and high specificity, or negative predictive value. Simply said, sensitivity is an ability of the marker to correctly determine patients with the disease, and specificity to correctly identify healthy subjects (Parikh et al., 2008). Besides these requirements, test for tumour markers should be simple, fast, cheap and easily obtainable, so they can be available for clinical examinations at any moment.

Tumour markers can be divided into three groups according to their utility – diagnostic, prognostic and predictive. The diagnostic marker should be able to distinguish patients with oncological disease from healthy subjects. Prognostic markers help to evaluate overall patient's condition and outcome, including progression of the disease and survival regardless of therapy. Predictive markers aim to predict patient's response to treatment and its effect, such as possible resistance including adverse side effects and overall patient's benefit from therapy (Mehta et al., 2010). In this chapter, several conventional and most common tumour markers utilised in clinical practice are described.

2.3.1. Tumour markers in tumour tissue

2.3.1.1. Genetic tumour markers

An important example of a gene marker is Kirsten rat sarcoma viral oncogene homolog (KRAS), which is a small GTPase protein active when bound to GTP. KRAS transmits downstream signal through MAPK signalling pathway (fig.4). A mutation in its gene *KRAS* may cause constitutive activation of the protein within the pathway leading to an

abnormal cell-proliferation. Therefore, analysis of mutated *KRAS* gene provides valuable information about patient's prognosis and prediction. More specifically, mutated KRAS was associated with worse prognosis in non-small cell lung cancer (NSCLC) patients with bone metastases (Lohinai et al., 2017), pancreatic ductal carcinoma (PDAC) (Sinn et al., 2014) and metastatic colorectal carcinoma (Zocche et al., 2015; Shindoh et al., 2016). There are ongoing clinical trials evaluating RNA interference therapy, called *siG12D-LODER*™, targeting most abundant *KRAS* mutations in pancreatic cancer, which are substitutions of glycine for aspartate in codon 12 (G12D) (Golan et al., 2015). Moreover, the presence of mutated *KRAS* is also a valuable predictor of treatment effect in patients treated with anti-EGFR therapy such as cetuximab (fig.5) (Karapetis et al., 2008).

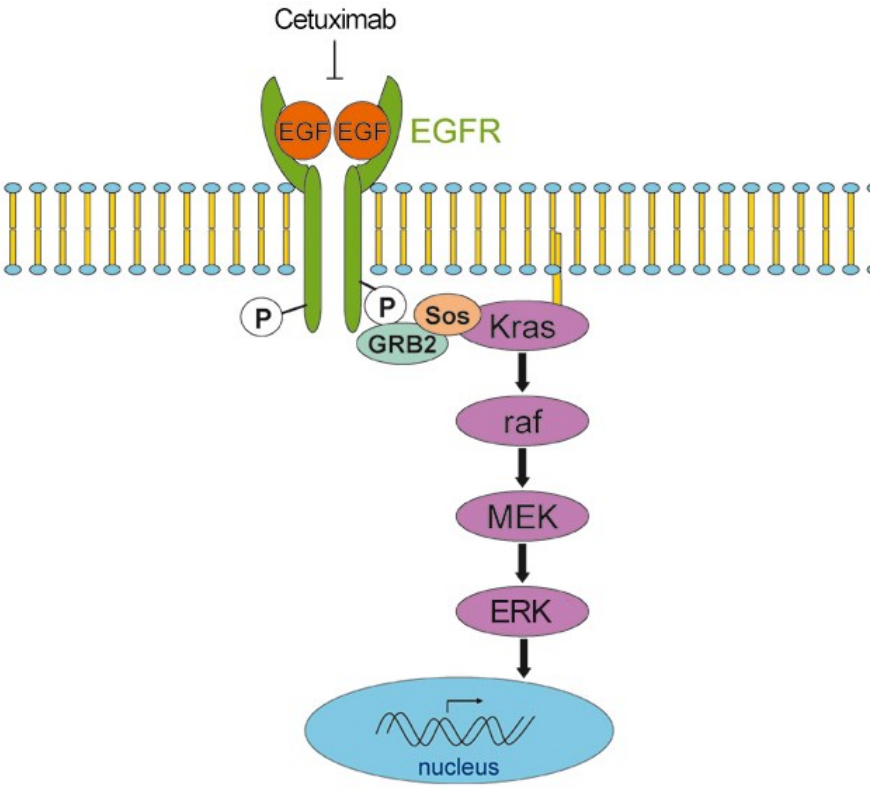

Figure 5. Scheme of Ras-Raf-MEK-ERK signalling pathway. Briefly described, epidermal growth factor (EGF), a ligand, binds to its receptor (EGFR) and activates its tyrosine-kinase activity, causing EGFR dimerisation and phosphorylation. After docking proteins (growth factor receptor-bound protein 2 - GRB2 and son of sevenless - SOS) are bound to phosphorylated EGFR, SOS exchange factor activates KRAS. Activated KRAS then activates BRAF kinase, which continues with activation and phosphorylation of MEK kinase, subsequently of ERK kinase, which in turn phosphorylates a transcription factor that regulates cell-proliferation in the nucleus. If a patient is treated with an inhibitor of EGFR (cetuximab), treatment may be ineffective because of constitutively active Ras protein downstream of the signalling pathway (Edited and adapted from Wicki et al., 2010).

Besides DNA markers, gene expression signatures are also important tumour markers. Oncotype DX® (Genomic Health Inc.) is one of the most commonly used expression panels. It is a clinically utilised prognostic and predictive assay focused on gene expression of breast, colon and prostate cancer. This test calculates the recurrence score (RS) based on reference-normalized gene expression of 16 tumour-related genes and 5 reference genes using real-time, also known as quantitative polymerase chain reaction (qPCR) (Wolmark et al., 2016; Fayanju et al., 2018). Another commercially available test is MammaPrint® (Agendia), available for breast carcinoma patients. It is a gene expression profiling assay, using microarray technology to evaluate gene expression of 70 tumour-related genes, involved in a cell cycle, angiogenesis, invasion and metastasis. This gene expression panel was able to classify breast carcinoma patients, irrespective of ER expression or lymph node metastasis status, into poor and good-prognosis signature, and accurately predict the risk of distant metastasis (Van't Veer et al., 2002; Fayanju et al., 2018)

2.3.1.2. Protein tumour markers

In contrast, an example of a protein marker test is called Mammostrat® (Applied Genomics), which relies on immunohistochemical assay tracking the expression of 5 different proteins such as tumour antigens, proteins of stress and hypoxia-inducible genes and genes involved in processes like cell cycle. These proteins are analysed in order to evaluate risk of recurrence of the disease in oestrogen (ER) positive and lymph node (LN) negative patients receiving hormonal therapy or chemotherapy, similarly to previous tests (Bartlett et al. 2010; Acs et al. 2013). Other commonly used protein markers that can be estimated from both tissue and serum are listed in chapter *2.4.1. Circulating protein tumour markers*.

2.4. Liquid biopsy

The term liquid biopsy represents a novel approach in oncological diagnostics. Its name already indicates that it is based on analysis of a sample obtained from body fluids, but mostly refers to blood, or more specifically, serum and plasma. The acquired sample then contains various types of molecules, vesicles and cells, which may be a source of important information about patient’s disease and condition. The merits of the liquid biopsy is a fact, that fraction of present DNA, RNA, proteins, extracellular vesicles, or even whole cells are shed from tumour, hence they contain features of the tumour cell they originate from. We

may then detect tumour-associated mutations of all kinds, chromosomal aberrations, epigenetic alterations, RNA expression profile and proteins, freely in circulation, bound to other proteins or included in extracellular vesicles such as exosomes (fig.6) (Diaz and Bardelli, 2014).

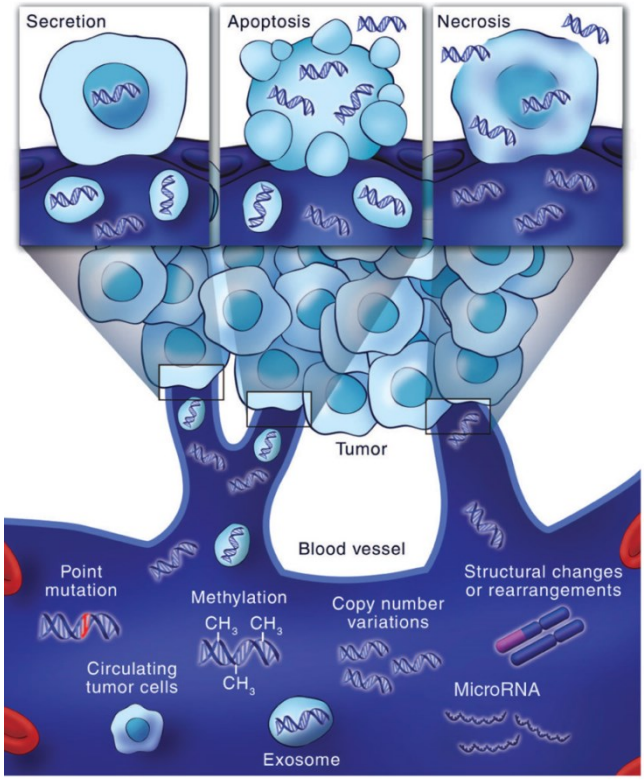

Figure 6. Scheme of various types of molecules, circulating tumour cells and exosomes present in the circulation of oncological patients (Adapted from Diaz and Bardelli, 2014).

The liquid biopsy possesses a couple of important advantages compared to the conventional biopsy. First, the conventional biopsy requires a surgical intervention, which can be uncomfortable or inconvenient for a particular subset of patients. Moreover, amount of acquired tumour cells in the sample depends on whether is biopsy performed during surgery or by fine and core needles, which allows removing only a small sample (Diaz and Bardelli, 2014). The main problem remains tumour heterogeneity as the neoplastic cells may genetically vary as a consequence of tumour evolution and carcinogenesis. New and new mutations are acquired during the proliferation of each cell, which can result in a presence of cellular subpopulations with different genetic makeup. Tumour heterogeneity can occur between cells within primary tumour, within particular metastasis, between individual metastases and among patients (Vogelstein et al., 2013). These genetically different subclones

could be missed and hence absent in the particular biopsied sample, therefore characterization of the disease might become inaccurate and biopsy has to be repeated (fig.7).

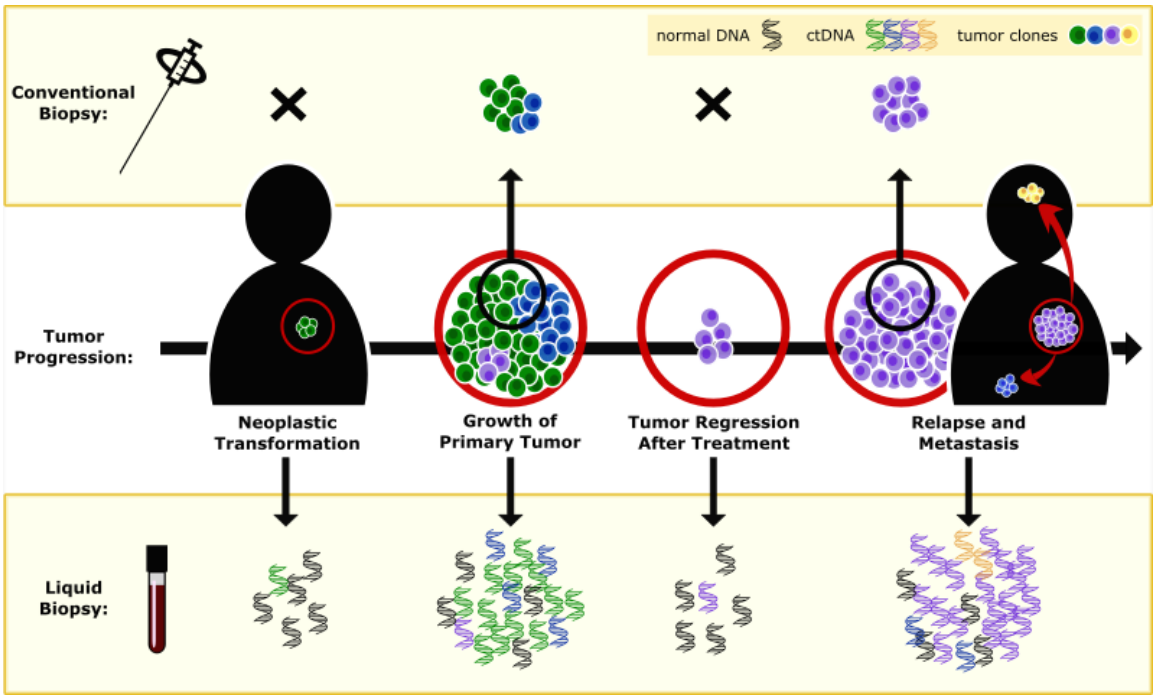

Figure 7. Representation of overcoming the limitation by using liquid biopsy compared to conventional biopsy (Adapted from www.genengnews.com/uncategorized/gen-roundup-liquid-biopsies-remain-wait-and-see-for-some-clinicians).

Liquid biopsy overcomes these problems, since proteins, cfDNA, cfRNA, CTCs and exosomes are released from all tumour lesions present in the body of the patient, including metastases, and correspond with tumour burden (Diehl et al., 2008). In some cases, the mutations characteristic for particular type of cancer can be detected only from blood, as some of the tumour subclones may not be included in the sampled tissue by biopsy (Rothé et al., 2014). In addition, genetic character of the primary tumour and metastatic lesion may differ, which can lead to underestimating malignancy of the disease or choosing inappropriate treatment. It is also important to say that liquid biopsy is faster and provides easily obtainable samples and non-invasive approach. It reflects real-time status of patient's condition, which can be useful for example in monitoring response to a therapy (Bettegowda et al., 2014). Individual approaches and their limitations are further described in the next chapters.

2.4.1. Circulating protein tumour markers in blood

Important type of tumour markers are protein markers that can found in body fluids, mainly in serum, or can be estimated in the tumour tissue. This group of markers involves membrane proteins, hormones and enzymes. Here are described a few of the most prevalent protein markers that are part of the clinical practice for many years and are utilised in everyday medicine (tab.2). However, nearly none of these biomarkers have sufficient specificity and sensitivity to serve as screening markers and the diagnosis has to be confirmed, for example by ultrasonography or biopsy (Bhatt et al., 2013).

Biomarker	Type	Application	Cancer	Possible false positivity	Citation
Carcino-embryonic antigen A (CEA)	oncofetal glycoprotein	diagnostic and prognostic	utilised for colorectal cancer, may be elevated in other cancers	liver diseases or in smokers	Bhatt et al., 2010
Cancer antigen 15-3 (CA15-3)	membrane glycoprotein mucin 1	diagnostic and prognostic	utilised for breast cancer, may be elevated in other cancers	hypothyroidism, hepatic dysfunction	Duffy, 2010
Carbohydrate Antigen 19-9 (CA19-9)	tetrasaccharide on mucin proteins	diagnostic and prognostic	utilised for pancreatic cancer, may be elevated in other gastrointestinal cancers	cirrhosis, jaundice, inflammatory diseases	Ballehaninna and Chamberlain, 2011
Cancer antigen 125 (CA125)	membrane glycoprotein mucin 16	diagnostic and prognostic	utilised for ovarian and fallopian tube cancer, may be elevated in other cancers	inflammatory and benign gynaecological conditions, pregnancy	Sjövall et al., 2002
Prostate-specific antigen (PSA)	serine protease	diagnostic and prognostic	prostate cancer	benign prostatic hyperplasia, infection or inflammation of the prostate gland	Gurel et al., 2014; Filella and Fernández-Galan, 2018

Table 2. List of most commonly utilised biomarkers.

2.4.2. Circulating tumour cells

Circulating tumour cells (CTCs) were first observed in 1869 in blood of a cancer patient as cells similar to those present in tumour (Ashworth, 1869; cited from Kapeleris et

al., 2018). Recently, CTCs gained a lot of attention because of their biological properties and behaviour. CTCs are cells shed from tumour mass that proceed into the vascular system and spread through the blood circulation in clusters or individually. These circulating cells may form metastasis after undergoing a process called mesenchymal-epithelial transition (fig.8) (Hosseini et al., 2016).

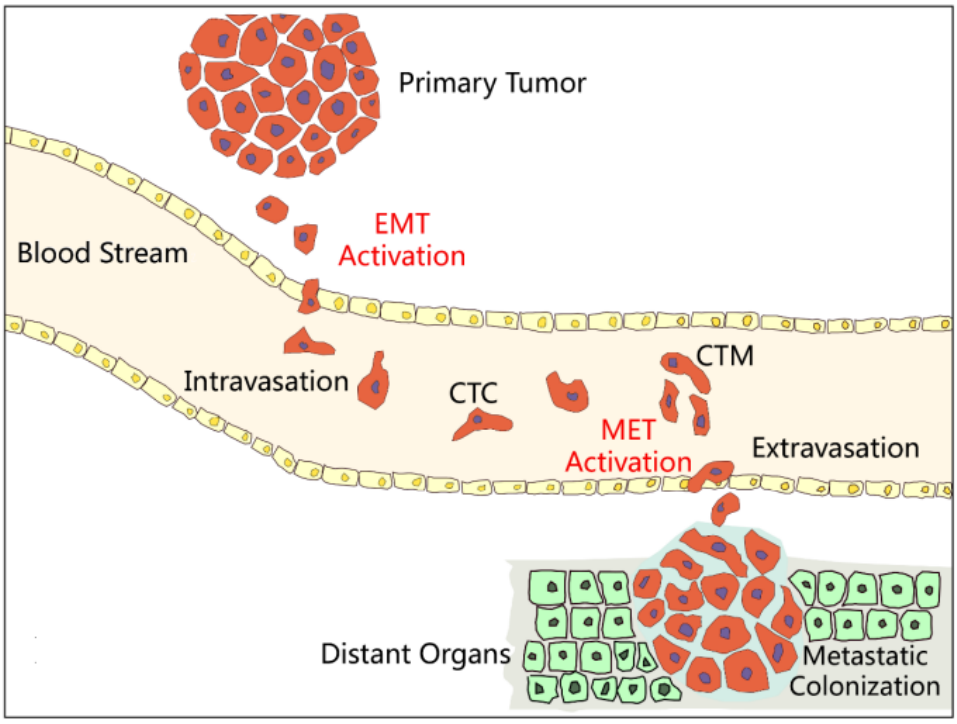

Figure 8. Scheme of emerging metastases. Tumour cells may undergo epithelial-mesenchymal transition (EMT), proceed into the blood and subsequently create new metastatic lesions through mesenchymal-epithelial transition (MET) (Adapted from Wu et al., 2017).

The presence and high number of CTCs in the blood of patients have been associated with a lot of important information such as poor overall survival in metastatic breast cancer and their detection predicted recurrence of disease earlier than imagining methods (Cristofanili et al., 2004; Budd et al., 2006). In addition, their expression pattern and mutations were able to predict metastatic status. For instance, Steiner et al. demonstrated that mutations discovered in CTC from colorectal cancer patient, such as mutation in *KRAS*, were not found in primary tumour. This observation could be very important in cases where patients receive ineffective anti-EGFR treatment because metastatic subclones have independently active EGFR signalling pathway (Steinert et al., 2014). A question remains how relevant is the different genotype of CTCs, or in other words, whether the mutations present in CTC could be acquired due to EMT or conditions in the environment of the circulation and if they truly represent the disease.

2.4.2.1. Detection of CTCs

Proper detection of CTCs is necessary for their enumeration but also for further genetic analysis and cultivation *in vitro*. For evaluation of patient's status and outcome, certain number of cells, for example, a threshold of ≥5 CTCs per 7.5 ml of blood, is being widely used (Cristofanilli et al., 2004). CTCs can be negatively or positively separated from healthy cells based on their morphological properties such as size (Jakabova et al., 2017), or according to their surface molecules. Most platforms detect the presence of epithelial cell adhesion molecule (EpCAM) molecules, cytokeratins (characteristic for epithelial cells) and the absence of CD45 (present on potentially contaminant lymphocytes). US Food and Drug Administration (FDA) approved using CellSearch in clinical practice, a method based on filtrating CTC cells by immunomagnetic beads and subsequently separating them with antibodies from lymphocytes. This platform, although FDA-approved, was however associated with some limitations, as many patients with metastatic diseases had undetectable CTC (Mego et al., 2011).

One of its problems is specificity to epithelial cells. EpCAM molecule can be downregulated due to EMT (Gorges et al., 2012), or even naturally in some cases, for example in thyroid cancer (Dent et al., 2016). Another limitation may be certain treatment, as lower number of CTCs was observed in patients with the progressive disease and bevacizumab (BVC) treatment. Besides tumour shrinkage, decreased expression of cytokeratins was observed in BVC-treated colorectal cancer cell lines and induced expression of EpCAM isoforms, without evidence of EMT (Nicolazzo et al., 2015). Many new platforms that are being evaluated, are trying to overcome these limitations. For example, CellSearch enriched by antibodies against another cytokeratine 20 improved CTC detection in colorectal cancer patients (Welinder et al., 2015). Combination of various commercialised platforms can also improve their detection ability (Gorges et al., 2016), or even using different and new technologies such as optic-fibres scanning technology (Ao et al., 2017). There is however a need for technological improvement of isolation and characterization of CTCs as it requires further research.

2.4.3. Exosomes

Exosomes are small, 30 to 100 nm vesicles, falling in a group of extracellular vesicles (EV). These vesicles are shed from most cells (including tumour cells) of an organism (Raposo et al., 1996), into body fluids environment, carrying tumour-associated molecules

and may contribute to the promotion of tumour progression (Muralidharan-Chari et al., 2011) and metastases (Peinado et al., 2013). Exosomes contain various important molecules and in circulation are protected from degradation due to the lipid bilayer membrane (Ge et al., 2014). The exosomal cargo includes mitochondrial DNA (Sansone et al., 2017), RNA molecules (Skog et al., 2012) and double-stranded DNA (Thakur et al., 2014), which contain a variety of information from a tumour which they originate from. Interestingly, they possess a potential immune-suppressing effect (Taylor and Gercel-Taylor, 2011), but can also transfer and deliver tumour-associated cargo, such as miRNA or mRNA into recipient cells (Valadi et al., 2007; Melo et al. 2014). There are also hypotheses suggesting that exosomes and their cargo from tumour cells may contribute to cell-to-cell communication (Pan et al., 2017). Analysing nucleic acids from exosomes that originate from tumour cells is therefore valuable contribution to overall liquid biopsy approach.

2.4.4. Circulating nucleic acids

Presence of circulating cell-free DNA (cfDNA) was first described in 1948 by French scientists Mandel and Métais in patients with oncological diseases and healthy individuals. Later, in 1977, Leon et al. discovered elevated levels of DNA in the serum of patients with several different types of cancer compared to healthy individuals. Moreover, they observed a decrease in DNA concentration after successful radiotherapy and increased or unchanged concentration in the cases of unsuccessful treatment, suggesting relapse of the disease or sign of poor prognosis (Leon et al., 1977). These findings encouraged researchers to continue in searching for nucleic acid-based and cancer-specific diagnostic, prognostic and predictive biomarkers, which would enhance the reliability of conventional tests and tumour markers. Another interesting example of DNA markers is of exogenous origin. For instance, viral HPV16 DNA was observed in the plasma of patients with cervical cancer by qPCR before treatment. After treatment, viral DNA was undetectable in 16 from 21 patients who responded well to the treatment (Yang et al., 2004).

2.4.4.1. Circulating transcriptome

After discovery of circulating cfDNA in the blood of patients, RNA also attracted attention as a potential specific biomarker. After identifying CEA mRNA in peripheral blood of pancreatic cancer patients (Funaki et al., 1996), interest in circulating transcriptome has risen. Another case of the presence of circulating cell-free RNA (cfRNA) was described

by Lo et al. in 1999, when they managed to detect latent gene transcripts of Epstein-Barr virus known to be present in the cells of nasopharyngeal carcinoma (NPC), EBER-1 RNA in plasma of 23 of 26 NPC patients (Lo et al., 1999). After that, Kopreski et al. detected human tyrosinase mRNA in the serum of 4 out of 6 patients with malignant melanoma (Kopreski et al., 1999). Later on, Koh et al. identified various tissue-specific RNA transcripts in plasma of four healthy women using technologies like RNA sequencing (RNA-seq) and microarray, pointing out an active RNA release into circulation, similarly to the case of DNA (Koh et al., 2014). Firstly, mRNA did not cause much interest because of its dubious stability due to elevated presence of RNA nucleases, or RNases, in blood of cancer patients (Reddi and Holland, 1976). However, it turned out that endogenous cfRNA is relatively stable in blood, suggesting its existence within particles such as exosomes and apoptotic bodies that protect it from RNAses, both in cancer patients and healthy individuals (Ng et al., 2002). After these discoveries, transcriptome started to be subject of many studies, mainly miRNome of cancer patients, which is described in the chapter *Cell-free miRNA in cancer research*.

2.4.4.2. CfRNA of coding genes in cancer

CfRNA of coding genes is still a subject of research. However, some interesting studies have been carried out, suggesting its promising potential. For instance, measuring overall cfRNA concentration together with a level of Telomere-specific reverse transcriptase mRNA (hTERT) (usually not detectable in healthy tissues but present in tumour tissues) in plasma of rectal cancer patients have been considered significant predictors of tumour response to preoperative chemoradiotherapy (Pucciarelli et al., 2012). Another example is detection of human epidermal growth factor receptor 2 (HER2) mRNA from the blood of breast cancer patients. Savino et al. performed qPCR assay observing higher HER2 mRNA levels in peripheral blood of HER2 positive patients compared to healthy controls with higher sensitivity and specificity than immunoenzymatic assay (Savino et al., 2009). Wu et al. also detected higher HER2 mRNA expression in peripheral blood of breast cancer patients; moreover, its levels were associated with response to neoadjuvant chemotherapy. However, no correlation to tumour size, grade, stage or ER and PR expression of primary tumour was observed. The group also claimed that further research is needed, as there are cases in which was not proven different expression of HER2 in blood of breast cancer patients (Owrangi et al., 2013; cited from Wu et al., 2018). Furthermore, concentration of mRNA in blood of

patients seems to be very low, thus using mRNAs as biomarkers in clinical practice remains a challenge (Imamura et al., 2016).

2.4.4.3. Non-coding cfRNA in cancer

About 70% to 90% of the human genome is transcribed into non-coding RNAs (ncRNAs) that do not code for any protein (Villegas and Zaphiropoulos, 2015). In respect to circulating biomarkers, most studies have focused on long non-coding RNA (lncRNA) and microRNA (miRNA), which may play an important role as diagnostic tools in the future.

2.4.4.4. Cell-free miRNA in cancer

MicroRNAs are short, approximately 18-22 bp long endogenous RNA molecules, which are known to regulate the expression of coding genes on post-transcriptional level (Lagos-Quintana et al., 2001). Leading strand of miRNA is bound to RNA-induced silencing complex (RISC) and subsequently silence target mRNA by binding to its 3'UTR region via specific seed sequence (Hammond et al., 2000). The dysregulation of miRNAs has been observed in cancerous tissues affecting tumour progression by repressing target genes involved in various signalling pathways. Additionally, the expression pattern of particular miRNAs allows to distinguish cancerous tissues from healthy ones, with greater accuracy than mRNA (Lu et al., 2005). For example, Lettlova et al. demonstrated that ESR1 mRNA is a direct target of miR-301a-3p in ERα positive breast cancer cells. Thus overexpression of this miRNA may lead to a decrease of oestrogenic signalling and promoting more invasive phenotype and oestrogen independence of breast tumour (Lettlova et al., 2018). Besides breast cancer, oncogenic activity of miR-301a-3p through various target genes has been also demonstrated in other tumours such as hepatocellular carcinoma (Hu et al., 2018), colorectal (Fang et al., 2015) and pancreatic cancer (Xia et al., 2015), proving its potential to serve as a biomarker.

Circulating miRNAs have gained their attention after discovering elevated levels of tumour-associated miRNAs in serum and plasma of patients with large B-cell lymphoma, proving their altered expression pattern not only in tumour tissues but also its detectability from the blood (Lawrie et al., 2008). Majority of miRNAs in circulation is bound to Argonaute2 protein (Ago2), which is normally a part of RISC complex in cytoplasm of cells (Arroyo et al., 2011). Besides Ago2, miRNAs can be found in high-density lipoproteins (Vickers et al., 2011) or in exosomes and vesicles (Valadi et al., 2007).

The diagnostic potential of circulating miRNA is one of the most promising features of this approach. Serum levels of miR-1290 had higher diagnostic accuracy than CA19-9 in distinguishing patients with low-stage pancreatic cancer from controls and pancreatic cancer from chronic pancreatitis or neuroendocrine tumours. Interestingly, levels of miR-1290 were not significantly different between patients with various size of the tumour (Li et al., 2013).

Another important aspect is the predictive value of miRNA. Zhu et al. were able to identify miR-222, which high plasma concentration was linked to poor response to non-adjuvant therapy in HR+/HER2- breast cancer. Furthermore, a post-chemotherapy increase of miR-222 was found to be present in insensitive patients (Zhu et al., 2018). There are a lot of other miRNAs, which make good candidates for further analysis as biomarkers. For instance, according to a review article by Komatsu et al., there have been identified 33 different up-regulated and 18 down-regulated circulating miRNAs in serum or plasma of patients suffering from gastric cancer. Some of them even acted oppositely in serum and in plasma (Komatsu et al., 2018).

Besides cell-free circulating miRNA, exosomal miRNA have drawn interest as a biomarker as well. However, vesicle-associated miRNA may behave differently. Tian et al. have not found any significant difference between quantified cell-free plasma miRNA and exosomes-derived miRNA of healthy individuals, whereas levels of two onco-miRNAs, miR-181b-5p and miR-21-5p, were higher in exosomes than in plasma of lung cancer patients (Tian et al., 2017). Findings like this suggest that miRNA level derived from exosomes should be interpreted differently from those in body fluids.

MiRNA has undoubtedly large potential, not only as a diagnostic tool, but also as a therapeutic, since phase I trial of a liposomal miR-34a mimic called MRX34 brings promising results for patients with advanced cancer by suppressing a number of oncogenes (Beg et al., 2017).

2.4.4.5. Long non-coding RNA in cancer research

LncRNAs are transcripts longer than approximately 200bp up to 100kb with specific expression patterns. These molecules possess various regulatory functions, enhancing or repressing various gene expressions by binding to specific sequences or acting as antisense RNAs and epigenetic modulators (Cheetham et al., 2013; Villegas and Zaphiropoulos, 2015). For example, lncRNA Hox transcript antisense intergenic RNA (HOTAIR) is sure one of lncRNAs worth mentioning. It has been demonstrated that HOTAIR interacts with

Polycomb Repressive Complex 2 (PCR2), which is known to alter chromatin state. Its overexpression is present in breast carcinoma tissues and is associated with metastases promotion and repression of tumour suppressor genes (PGR, HOXD10, proto-cadherin gene family) in breast cancer cell lines (Rinn et al., 2007). In regards to circulating lncRNA, increased levels of serum HOTAIR were found in patients of breast cancer compared to controls. Moreover, its changing levels were shown to predict response to neoadjuvant chemotherapy of breast cancer patients (Lv et al., 2018).

Metastasis associated in lung adenocarcinoma transcript 1 (MALAT-1) is an approximately 8000 nt long lncRNA expressed in healthy tissues as well as in cells of NSCLC and other types of cancers, with a role in variety of cellular functions such as proliferation, cell death and cycle, migration, angiogenesis, tumorigenesis and is involved in many signalling pathways and regulations of gene expressions (Li et al., 2018). High level of MALAT-1 expression in tissue and serum was demonstrated to occur in breast cancer patients (Miao et al. 2016). Zidan et al. have reported MALAT-1 positively correlated with oestrogen receptor, stage of the tumour and lymph node status. The group also compared sensitivity and specificity of MALAT-1 (83.7% and 81.2%) with those of protein marker CA15-3 (77.5% and 82.5%), enhancing the accuracy of both markers combined (Zidan et al., 2018). On the other hand, numerous of researches reported that knockout or inactivation of MALAT-1 in cells lines or in transgenic mouse models promotes metastases, and overexpression suppressed tumour growth for example by interacting with specific miRNAs such as miR-124 (Feng et al., 2016) and miR-155 (Cao et al., 2016). Findings like these suggest that this field of research is still at the beginning; however, many interesting lncRNAs were identified as promising candidate biomarkers in the future.

2.5. Circulating cell-free DNA

As previously said, the majority of cfDNA is approximately 180-200 bp long DNA circulating in the blood or packed in exosomes. The fraction of cfDNA that is derived from tumour cells is termed circulating tumour DNA (ctDNA) and contains various mutations such as tumour progression-associated mutations, gene amplifications or copy number variations (CNV), epigenetic modifications such as hypermethylation of gene promoters, loss of heterozygosity and microsatellite alterations (fig.9). Additionally, half-life of cfDNA in blood is between 16 min and 2.5 h providing real-time insight into the patient's current status (Diehl et al., 2008). Besides the ctDNA fraction, other parameters of cfDNA have been proven to provide valuable information. Monitoring dynamic changes in levels of cfDNA

and ctDNA, size of its fragments and cfDNA integrity (cfDI) contribute to its value as a biomarker, as described in the following chapters.

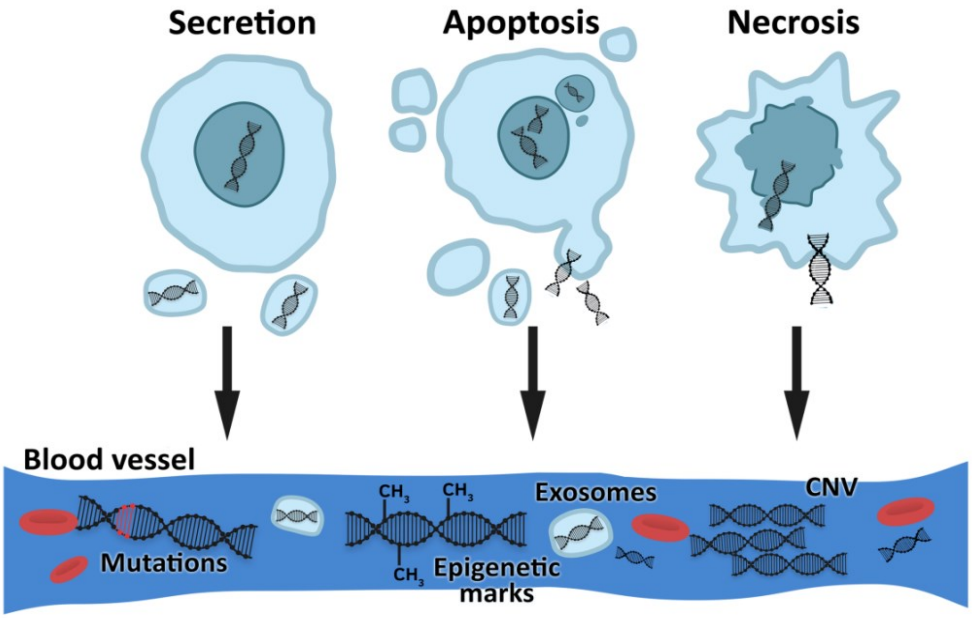

Figure 9. Scheme of origin and properties of cfDNA. CfDNA provides real-time information about patient's condition by monitoring cfDNA concentration, integrity, specific mutations, copy number variations (CNV), epigenetic marks, loss of heterozygosity etc.

There are three hypothetic mechanisms of a release of nucleic acid: Apoptosis, necrosis and secretion. It has been known that cultivated human lymphocytes actively release certain amount of DNA into the surrounding environment. The same amount of DNA was found in medium independently of incubation duration; in addition, cell death rate had no effect on amount of DNA in media, suggesting its active release (Anker et al., 1975). In 1994, Abolhassani et al. have demonstrated a release of newly synthetised DNA from human promyelocytic leukemic cell line (HL-60) to surrounding media, but could not elucidate the mechanism (Abolhassani et al., 1994). Moreover, when visualised on gel electrophoresis, DNA from pancreatic cancer patients' plasma and serum showed ladder-like pattern resembling apoptotic DNA from healthy individuals (Giacona et al., 1998; cited from Stroun et al., 2001). Based on these findings, later in 2000, Halicka et al. have proven the presence of RNA and DNA packed separately in apoptotic bodies using immunohistochemistry (IHC) in HL-60 and MCF-7 cell lines (Halicka et al., 2000).

Finally, Jahr et al. demonstrated the presence of fragments of approximately 180 bp, but also fragments of two, three or four times of this length. Presence of such fragments

suggested the mono, di or tri-nucleosomal form of the DNA, resembling the apoptotic ladder pattern. This important finding was strongly indicating occurrence of the fragments within nucleosomes. Moreover, fragments as long as ~ 10 000 bp were also observed, implying their origin from necrotic cells (fig.10) (Jahr et al., 2001). Such observations have sparked an interest in origin of DNA fragments present in blood of cancer patients. A hypothesis, that long cfDNA fragments are of necrotic origin and short ones from healthy cells is described in more detail in the chapter *2.5.6.2. Non-specific ctDNA measurement*.

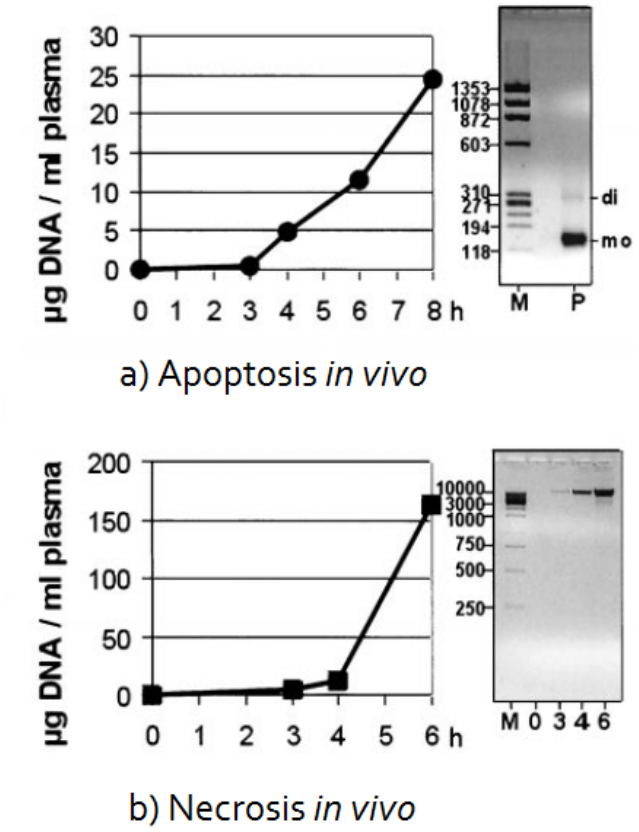

Figure 10. Demonstration of a rising DNA concentration in plasma after inducing cell death in mice. a) Apoptosis was induced with an injection of anti-CD95 antibody; visualisation exhibits apoptotic patter with mono and di-nucleosomal fragments. b) Necrosis was induced with acetaminophen; long fragments characteristic for necrosis were observed. DNA concentration was analysed using qPCR. (Jahr et al. 2001). di = dinucleosomal; mo = mononucleosomal fragments.

2.5.1. Loss of heterozygosity and microsatellite alterations

The loss of heterozygosity (LOH) refers to loss of one allele of a gene. Regarding to tumorigenesis, it means loss of remaining wild-type allele, often of a tumour suppressor gene, as the other one is mutated. Microsatellites are short tandemly repeated DNA sequences,

which can differ in a number of repeats and lead to high frequency of microsatellite instability (MSI) as a result of malfunctioning Mismatch Repair System of DNA (MMR) (Elshimali et al., 2013). Both LOH and MSI are present in various types of cancers and are often present in characteristic loci. MSI, often present in colorectal patients, was associated with a better prognosis than in patients without MSI (Nawroz et al., 1996; cited from Qin et al. 2016). Another example is adenomatous polyposis coli (APC), which is one of the genes screened for present of MSI and LOH, acting as tumour suppressor. Presence of mutation and instability in such genes can lead to its inactivation and provide invasiveness (Kamat et al. 2013). Schwarzenbach et al. detected LOH in 8 microsatellite markers mapping to tumour suppressor genes of breast cancer patients plasma. High frequencies of LOH in 5 of these markers were associated with more aggressive phenotype, and LOH at marker D12S1725 mapping to cyclin D2 has correlated with shorter survival. Moreover, in HER2-positive patients were also observed LOH at marker D17S855 mapping to well-known gene BRCA1 (Schwarzenbach et al., 2012).

Pembrolizumab is a treatment working like inhibitor of PD-1/PD-L signalling pathway of innate immunity, which is known to provide inhibitory signals as T-cell inhibition and inactivation, unwelcomed in fighting against cancer progression (Villasboas and Ansell, 2016). It is a FDA-approved treatment for various types of solid tumour patients with detected microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) biomarker (www.fda.gov/newsevents/newsroom/pressannouncements/ucm560167.htm). Detection of MSI from cfDNA can, therefore, provide helpful modality in case of considering this treatment (Feng et al., 2018). MSI and LOH can be assessed in plasma of patients for example using PCR with fluorescence-labelled primers for specific microsatellite markers (Schwarzenbach et al., 2012) or by sequencing (Mayrhofer et al., 2018).

2.5.2. Epigenetic modifications in cfDNA

Aberrant methylation of DNA sequences, such as promoters of tumour suppressor genes or other tumour-specific modifications are detectable from the plasma of patients. Interesting example is Septin9 (SEPT9) gene, of which function is involved in remodelling cytoskelet and cytokinesis. Methylation of this gene has been associated with tumorigenesis and is usually present in CRC patients (Warren et al., 2011). Commercially available Epi proColon 2.0 is a blood-based test that detects presence of mSEPT9 cfDNA from plasma using methylation-specific PCR and is widely used in clinical practice in the United States.

However, this test cannot serve as diagnostic marker alone and has to be verified with endoscopy (Lamb and Dhillon, 2017).

As previously described, cfDNA in the blood occurs wrapped around nucleosomes. Besides DNA methylation, histones within nucleosomes can possess specific histone modifications (HM), which affect the rate of gene transcription through chromatin condensation and decondensation. For example, H3K9me3 (trimethyl of 9 lysine on histone H3) and H4K20me3 (trimethyl of 20 lysine of histone H4) marks are usually present in stably silenced DNA called constitutive heterochromatin, in which frequently occur tandem repeats and interspersed elements and is often associated with transcriptional repression (Barski et al., 2007). Gezer et al. measured the rate of three HM in plasma of CRC patients: H3K9me3, H4K20me3 and H3K27me3. They observed a significantly decreased the amount of H3K27me3 and H4K20me3 marks in cancer patients compared to healthy controls estimated by IHC. Histone modification could serve as source of the complementary tumour-specific marker. However, the group also reported contradictory results from IHC and chromatin immunoprecipitation (CHIp) assays, and the need for additional research (Gezer et al., 2015).

2.5.3. Genetic alterations

The mutational profile of the tumour cells includes large changes like chromosomal aberrations, but also smaller mutations present in DNA sequences. CtDNA containing such alterations is a valuable tool for liquid biopsy, but also for non-invasive prenatal testing. Chromosomal aberrations in cfDNA are investigated mainly for prenatal screening purposes, analysing foetal cell-free DNA for aneuploidies and copy number variations (CNVs). These aberrations can be identified using techniques like next-generation sequencing (NGS) and sensitive PCR-assays and can reveal genetic disability such as trisomy of the foetus (Pescia et al., 2017). CNVs may be identified from ctDNA of cancer patients, as they are important part of carcinogenesis and tumour progression (Wang et al., 2015). Li et al. examined aberrations in colorectal cancer patients within cancer-specific chromosomal regions and detected amplifications and CNV accumulations of genes involved in DNA repair, signalling pathways (such as MAP or JAK/STAT signalling) and cell cycle. However, it is not possible to evaluate the CNVs' epigenetic status, which may be important for the colorectal disease. Monitoring CNV in these stages can serve as complementing information to aid overall characteristics of the tumour but is not ready to be a screening tool yet (Li et al., 2017).

Tracking of the cancer-specific mutations is probably the most exploited potential of cfDNA. Identifying such somatic alterations from plasma distinguish the tumour-derived ctDNA fraction from overall cfDNA and may serve as sensitive but also a specific biomarker for advanced stages of cancer (Newman et al., 2014). Predictive potential of ctDNA is surely one of its most important features. Analysis of ctDNA can provide real-time monitoring of patient's benefit from treatment. For instance, Mohan et al. used whole-genome sequencing of CRC patients' plasma for monitoring of newly acquired mutations after induction of anti-EGFR therapy. Acquired resistance to anti-EGFR therapy was associated with gained *KRAS* gene amplification detected in ctDNA of these patients (Mohan et al., 2014). Another evidence of the predictive value of ctDNA is commercialised FDA-approved test termed The Cobas® EGFR Mutation Test v2. It is a qPCR-based detection panel for 42 various mutations in *EGFR* gene from the plasma of NSCLC patients, in order to select candidate patients for anti-EGFR treatment and overcome possible resistance, as mutations in *EGFR* are observed in 15 to 30% of patients with NSCLC (Brown, 2016). However, there are also studies that failed to associate the *KRAS* mutation in ctDNA with prognosis or selection of appropriate patients for anti-EGFR therapy, specifically in rectal cancer (Sclafani et al., 2018). The detection of the mutations is also a strategy how to quantify specifically ctDNA, in contrast to quantifications of whole cfDNA non-specifically. Further discussion on this topic is held in chapter *2.5.6.1. Specific quantification of ctDNA.*

2.5.4. Circulating DNA concentration

Already in 1994, Sorenson et al. observed increased concentration of cfDNA in pancreatic patients. In addition, they were able to detect mutated *KRAS* sequence in plasma of these patients (Sorenson et al., 1994). Since then, an astronomical number of papers was published demonstrating various kinds of approaches and techniques for detection of cfDNA and ctDNA concentrations in plasma and serum of patients. Concentration can be measured either specifically, or non-specifically. The difference lies in non-specific measurement of gross amount of the cfDNA copies in blood, while the concentration of the ctDNA fraction can be measured by tracking the amount of specific target mutations present only in cancer-derived ctDNA. Here are described few cases of monitoring the circulating DNA, in which interesting behaviour of cfDNA was observed.

2.5.5. CfDNA concentration measurement

The concentration of the whole cfDNA can be assessed using several strategies. Firstly, plasma or serum cfDNA can be assessed using qPCR technology. The cfDNA fragments can be then amplified using primers for house-keeping genes or highly abundant DNA repetitive elements, such as Alu or LINE-1 repetitions (Madhavan et al., 2014), β-actin (ACTB) (Szpechcinski et al., 2016) or human telomerase reverse transcriptase gene (hTERT) (Mazurek et al., 2013). Umetani et al. claimed that detection limit of the Alu-based qPCR assay reaches to 0.01 pg of DNA (Umetani et al., 2006).

Another method for relatively simple cfDNA quantification with comparable sensitivity to qPCR is PicoGreen assay (Chiminqgi et al., 2007). Picogreen is a fluorescent dye with high affinity to dsDNA with detection limit as low as 25 pg/µl (Holden et al., 2009). What might be its limitation is a possibility that fluorometric assays for cfDNA concentration measurement can be affected by presence of carrier RNA, used for extraction by several isolation kits. The amount of cfDNA may be then overestimated (Bali et al., 2014). Other possible limitations based on DNA fragment sizes are further discussed in chapter *6 Discussion*.

Ultraviolet-visible spectrophotometry, e.g., using NanoDrop, is another method used to quantify cfDNA. Surprisingly, Park et al. have observed a negative correlation of plasma cfDNA levels with treatment outcomes, in patients with hepatocellular cancer (HCC) treated with radiotherapy using NanoDrop 2000 (Park et al., 2018), which may raise questions about the accuracy of this modality in regards to cfDNA. General disadvantage of this absorbance method is low sensitivity of the assay and contribution of single-stranded nucleic acids, proteins and nucleotides to overall absorbance value (Georgiou and Papapostolou, 2006).

Digital PCR (dPCR) is generally considered as one of the most sensitive approaches for cfDNA quantification (Whale et al., 2017). Droplet digital PCR (ddPCR) is a method using dPCR technology based on water-oil emulsion droplets. As its PCR-based assay, quantification of overall cfDNA can be assessed by amplifying of the house-keeping target gene, such as RNase P (Earl et al., 2015; Garcia-Murillas et al., 2015), as it is conducted in this study. However, quantification of ctDNA fraction has probably gained more attention by the specificity of this fashion, which is proven by a number of publications focusing on the dynamics of ctDNA levels, discussed in the next chapter.

2.5.6. CtDNA concentration measurement

2.5.6.1. Specific quantification of ctDNA

The clinical potential of ctDNA as a biomarker can be explained from an exemplary study, such as from Dawson et al. The group tracked ctDNA dynamics in 52 metastatic breast cancer patients during treatment by means of tumour-specific mutations, which were previously identified from tumour tissue of these patients. Levels of ctDNA were changing depending on treatment as seen in figure 11. CtDNA levels and mutations were estimated using sensitive modalities such as targeted sequencing and ddPCR.

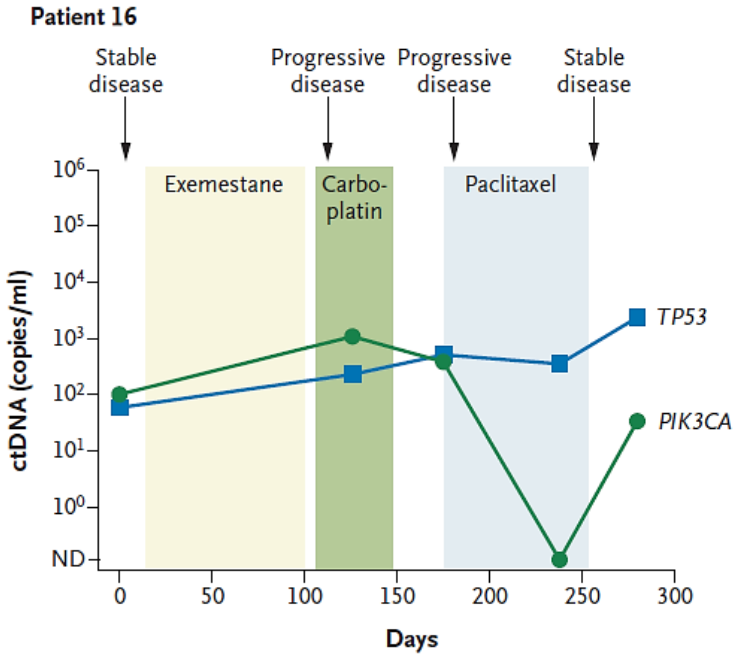

Figure 11. Level of ctDNA changing according to the treatment. Two different mutations were monitored from plasma ctDNA. Point mutations in *PIK3CA* and *TP53* were assessed using tagged-amplicon deep sequencing. In this patient, mutated *TP53* did not decrease even after inducing treatment, indicating the presence of the tumour even after the establishment of treatment. Moreover, the *TP53* gene mutation was detected only in the patient's plasma (Adapted from Dawson et al., 2013).

Furthermore, in 10 of 19 patients (53%), an increase in levels of ctDNA was detected before evaluating the disease as progressive by imaging methods (fig.12, patient 17). Additionally, this group compared the sensitivity of ctDNA to the conventional tumour marker CA 15-3 and to CTCs in patients' plasma.

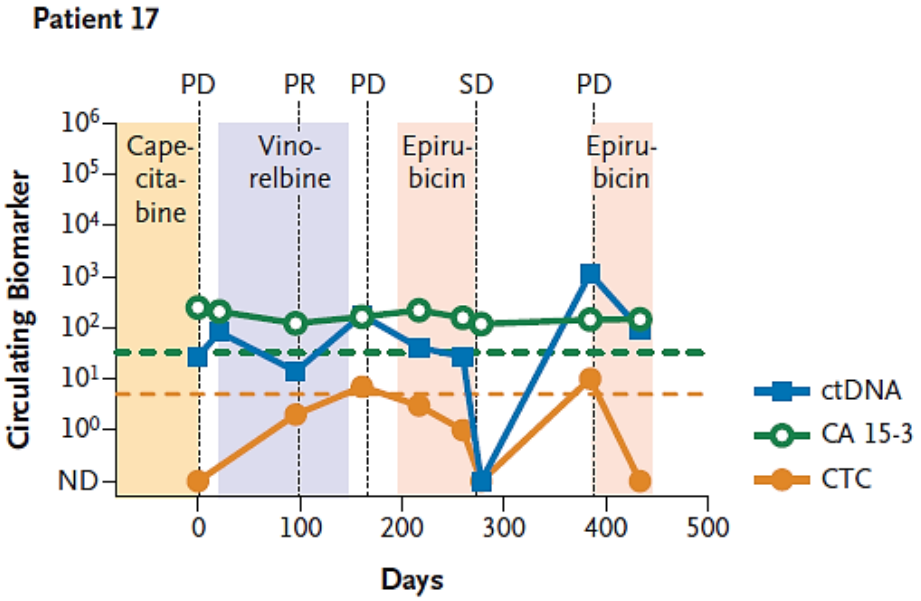

Figure 12. Changing levels of ctDNA, tumour marker CA15-3 and CTCs in patients suffering from metastatic breast cancer. The level of ctDNA after ended epirubicin treatment has risen, which indicates the progress of the disease. SD = stable disease; PR = partial response; PD = progressive disease. ctDNA is shown as a number of copies/ml of plasma; Orange line represents 5 CTCs/7.5ml of blood; green lines represent threshold of CA15-3 32.4 U /ml (Adapted from Dawson et al., 2013).

CA15-3 was detectable in 27 patients. Elevated levels of CA15-3 were observed in 21 of the 27 women (sensitivity 78%), whereas ctDNA levels in 26 of these 27 women (sensitivity 96%). Overall calculated sensitivity of ctDNA vs. CA15-3 in the study was 85% vs. 59%. Moreover, 27 of 43 samples with no increased CA15-3 had detectable levels of ctDNA. CTCs were detected in 26 of the 30 women (87%), whereas ctDNA was detectable in 29 of the 30 women (97%). Additionally, levels of ctDNA were observed in 50 plasma samples with no detectable CTCs, suggesting that these two biomarkers behave independently. The group calculated the sensitivity of ctDNA versus CTCs to 90% vs. 67%. Regarding prognosis, patients with 2000 and more copies of ctDNA were associated with a worse prognosis. Dawson et al. stated that ctDNA level dynamics provided fast and valuable prognostic information superior to CA15-3 and CTCs (Dawson et al., 2013).

Other interesting studies using tracking of ctDNA as a biomarker are listed in table 3. Another observation worth mentioning was demonstrated in patient with brain metastasis who had no detectable mutations in their plasma. This result suggests that blood-brain barrier blocks ctDNA release into body circulation (Garcia-Murillas et al. 2015). Additionally, this hypothesis is supported by earlier research demonstrating low ctDNA levels present in patients with primary brain cancer (Bettegowda et al., 2014).

	Patients	Screened mutations	Application	Platform
Diehl et al., 2008	metastatic colorectal cancer patients	*KRAS,APC, PIK3CA, TP53*	response to chemotherapy, prediction of recurrent diseases	BEAMing
Garcia-Murillas et al., 2015	early breast cancer patients	*TP53, PIK3CA*	prediction of relapse, identification of tumour-specific mutations in indication of a minimal residual disease before clinical relapse	ddPCR, massive parallel sequencing
Bettegowda et al., 2014	metastatic colorectal cancer patients	*KRAS, NRAS, BRAF EGFR, PIK3CA*	detection of acquired resistance mutations to anti-EGFR therapy	Targeted sequencing, exome sequencing, whole-genome sequencing
Riva et al., 2017	triple-negative breast cancer patients	*TP53*	response to neoadjuvant chemotherapy, detection of minimal residual disease after surgery	ddPCR

Table 3. Examples of utilisation of ctDNA as a biomarker

All these results suggest the very promising potential of ctDNA and its contribution to personalised patient approach; however, contradictory results were reported. Riva et al. have not managed to detect mutation of *TP53* in plasma after surgery of patients of triple-negative breast cancer, even though they reported latter metastatic relapse in few patients (Riva et al., 2017). Furthermore, they assessed overall cfDNA plasma concentration by means of LINE-1 qPCR assay, and levels of ctDNA by tracking *TP53* mutation using ddPCR in plasma. Interestingly, the concentration of cfDNA increased whereas ctDNA levels decreased during neoadjuvant chemotherapy, possibly explained by the death of healthy cells due to chemotherapy and the release of their DNA into surroundings (Butler et al., 2019). No correlation was observed between ctDNA and cfDNA. Only one patient experiencing tumour progression during therapy had rising levels of ctDNA (Riva et al., 2017). These observations suggest that mentioned approaches for cfDNA analysis are different and should be interpreted independently.

2.5.6.2. Non-specific ctDNA measurement

Cell-free DNA integrity index (cfDI) is a parameter that represents non-specific quantification of the tumour fragments of ctDNA. It is based on a hypothesis, that longer fragments of cfDNA (which length can reach several kilobases) originate in necrotic cells, whereas the shorter fragments (around 180 bp) are derived from apoptotic cells. Therefore, there is an assumption that cfDNA of healthy individuals originate mainly from apoptosis, while in oncological patients from both apoptosis and necrosis (Umetani et al., 2006). CfDI is then estimated as a ratio of longer fragments to all fragments detected in blood. Many studies already used Alu-based qPCR, not only to evaluate cfDNA concentration, but also cfDI. Tested assay in this study was designed following research by Umetani et al. from 2006, which was based on absolute quantification of short and long cfDNA fragments using two pairs of primers for Alu repetitive DNA elements. ALU-115 primers are designed to yield short PCR products (115 bp) and could be primarily used for cfDNA quantification. On the other hand, amplification efficiency of longer fragments is more related to cfDNA quality, thus ALU-247 primers are used for testing DNA integrity. Because annealing sites of ALU-115 primers are located in 247 bp fragment of ALU-247 primers, the amount of short and long fragments, expressed as their ratio, is used to determine DNA integrity (fig.13) (Umetani et al., 2006).

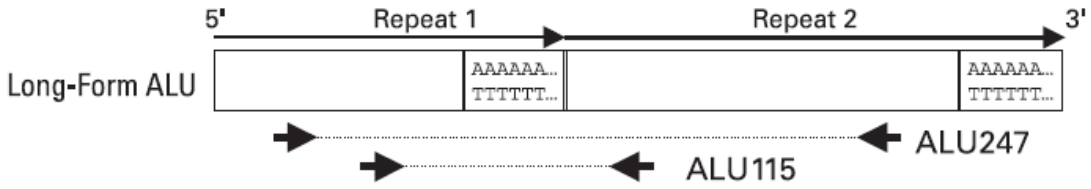

Figure 13. Annealing sites of Alu-based primers. Annealing sites of ALU-115 primers are situated in annealing sites for ALU-247, which ensures that ALU-115 amplify all the fragments, whereas ALU-247 only the longer ones (Adapted from Umetani et al., 2006).

Besides Alu-assay, other primers for long and short fragments that can quantify cfDNA can also serve to assess cfDI, such as primers for LINE DNA repetitive elements, *ACTB* and *TERT* gene, as mentioned in *2.5.5. CfDNA concentration measurement*. Many studies already reported elevated cfDI values in patients with cancer compared to healthy individuals (Umetani et al., 2006; Soliman et al., 2017; Sobhani et al. 2018). On the other hand, there are works that reported a decrease in cfDI with tumour progression. Further analysis and discussion is carried out in chapter *6 Discussion.*

2.5.6.3. Preview of ctDNA detection strategies

For specific tracking mutations in ctDNA, qPCR using mutation-specific TaqMan probes is one of the most used platforms. However, in recent years, modern technologies have taken over. Methods based on PCR assay such as digital PCR (dPCR), BEAMing (Beads, Emulsions, Amplification and Magnetics) and NGS are used in nowadays papers thank to their high specificity and sensitivity compared to conventional qPCR technique. Previous studies claimed that ddPCR and NGS technologies correlated very well and had similar accuracy (Diehl et al., 2012; Dawson et al., 2013; Garcia-Murillas et al., 2015). However, even these modalities have their limitations as there were reported cases of possible unsuccessful detection of ctDNA or present mutations (Riva et al., 2017; Cabel et al. 2019). The greatest obstacle to overcome is the small amount of the ctDNA fraction and competing wild-type cfDNA alleles. Moreover, a lot of papers using NGS technologies for detection of somatic nucleotide variants rely on computational techniques such as allele fraction (AF). AF determines the percentage of „alternative reads“, the reads with different nucleotides in sites of interest compared to the reference genome. Loci with AF under the set threshold are excluded even though alternative reads are detected. For example, set threshold of 1% represents that sites with 10 or less alternative reads are ruled out at the sequencing depth of 1000×. To ensure high percentage of true positives, many studies set the threshold strictly to avoid false-positive mutation calls or noise caused by sequencing or PCR errors, effect of CNV etc. However, very small amounts of ctDNA may fall under AF threshold and therefore a number of true mutations could be removed (Tian et al., 2019). Considering findings like this, many new and enhanced modified NGS technologies and algorithms have been and are being developed. For example, Bias-corrected targeted NGS was able to identify mutations with as low as 0.4% allelic frequency and with 100% specificity in NSCLC patients, using multifunctional adaptors that enable identifying unique sequence clones. This technique was able to perform highly sensitive genotyping without false-positive results due to PCR artefacts (Paweletz et al., 2016). A lot of other NGS platforms are utilised in research with specific advantages and disadvantages, some of which are listed in the table 4. The main advantage of NGS compared to PCR-based technologies is the possibility of detection of tumour profiling panel with many genes. Multiplex ddPCR can be also performed but in a limited dimension. Of course, lower specificity and sensitivity of more conventional assays such as qPCR is a disadvantage, but the cost and difficult data analyses of modern platforms should be also considered.

	Platform	Sensitivity	Specificity	Number of targets	Alteration	Limitations
Next-gen. sequencing	Deep sequencing (>10,000×)	0.02%	80–90%	Panel	Genome-wide copy number changes	Unable to detect rearrangements without assay customization
	TAm-Seq	0.02%	99.9997%	Panel	Known point mutations	Detects only known mutations
	CAPP-Seq	0.004%	>99.99%	Panel	Known point mutations rearrangements CNV	needs high cfDNA input; detects only known mutations
	Bias-Corrected Targeted NGS	>0.4%	100%	Panel	Known point mutations rearrangements CNV	-
Digital-PCR	ddPCR	0.1%	100%	1 - 3	Known point mutations	Detects specific genomic loci; limited in multiplexing
	BEAMing	0.01%	100%	1 - 20	Known point mutations	Detects only known mutations
Real-Time PCR	AS-PCR	1%	98%	1	Known point mutations	Low sensitivity detects known mutations
	MS-PCR	0.62%	100%	1	Known methylation sites	Detects only specific CpG islands

Table 4. List of common used approaches.

Notes: TAm-Seq = Tagged-amplicon deep sequencing; CAPP-Seq = Cancer Personalized Profiling by deep sequencing; ddPCR = Droplet Digital PCR; BEAMing = Beads, Emulsion, Amplification and Magnetics; AS-PCR = Allele-specific amplification; MS-PCR = methylation-specific PCR (Edited and adapted from review article by Elazezy and Joosse, 2018).

2.5.6.4. Limitations

Too little amounts of ctDNA are apparently one of the main reasons of discordance between studies and remain a great technological challenge. Besides measurement techniques and their limitations, other reasons for inaccurate cfDNA and ctDNA detection rate are differences between blood processing, storage ways, extraction methods and isolation protocol. For instance, the storage of cfDNA differs among laboratories. Kumar et al. found that 36% of 84 different laboratories stored plasma at 4°C before cfDNA isolation, and 41% at -80°C (Kumar et al., 2018). Although freezing of cfDNA for longer periods is recommended, cfDNA degradation in plasma as well as of extracted cfDNA after one year of storage at -20°C and -80°C was reported (Sozzi et al., 2005). Interesting comparative study

claimed, that 53 different laboratories extracted identical spiked plasma cfDNA samples with surprisingly different cfDNA yields – measured concentrations ranged from 2.87 to 224.02 ng/ml (Malentacchi et al., 2015). All these aspects apparently play important role in cfDNA or ctDNA quality and quantity.

What remains still debatable is choosing between serum and plasma. There have been studies proving a higher amount of cfDNA in serum, but also that serum can be prone to contamination by lymphocyte DNA due to delayed processing (Chan et al., 2005). Another reason may be a clotting process leading to lysis of white blood cells. There is thus a chance of contamination by germline DNA from hematopoetic cells (Chang et al., 2017). Netosis or neutrophil extracellular trap has also been proposed as a reason for a significantly higher amount of cfDNA in serum; Zinkova et al. hypothesised that source of cfDNA in serum could be genomic DNA of activated neutrophils (Zinkova et al., 2017). Kumar et al. claimed, that serum was a preferable source of cfDNA until 2010, whereas plasma is the preferred source for analyses of cfDNA and ctDNA since then (Kumar et al. 2018). Additionally, measurement platforms and their specificity and sensitivity differ, which is another important aspect to consider during cfDNA analysis. Additional discussion can be found in chapter *6 Discussion.*

3 Aims

The first aim of the thesis is to select and optimize the methods for cell-free DNA extraction from plasma and serum samples and methods for quantification of cell-free DNA and circulating tumour DNA.

NORGEN Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit (Norgen Biotek) and QIAamp® Circulating Nucleic Acid (QIAamp CNA kit for short) were used for cfDNA extraction from plasma and serum samples and subsequently compared. Quant-iT™ PicoGreen™ dsDNA Assay Kit, absolute quantification using real-time PCR, and droplet-digital PCR were used for evaluation of an appropriate quantification method of cfDNA. Finally, cfDNA integrity was used to estimate ctDNA portion in cfDNA samples.

The second aim of the thesis is to verify whether the levels of the cfDNA and ctDNA in blood of patients with different types of cancer differ in comparison to healthy individuals and whether these two parameters depend on the stage of the disease. Blood samples from patients with breast, ovarian, colorectal, and pancreatic carcinomas, blood samples from patients with benign diagnose of breast and ovary and samples from healthy volunteers were utilised. Obtained levels of cfDNA concentration and cfDNA integrity were evaluated with clinical data of patients.

Chapters *Methods* and *Results* are therefore divided into *Optimisation part*, where selecting and validation of methods and protocols is described, and *Measurement part*, where cfDNA concentration and integrity of patients' samples are evaluated using previously optimized protocols.

4 Material and methods

4.1. Used chemicals

6x Loading Dye Solution (Fermentas)
Absolute ethanol (PENTA s.r.o.)
ACTB 117/382 primers (Invitrogen™)
ALU 111/260 primers (Sigma-Aldrich)
ALU 115/247 primers (Invitrogen™)
ddPCR Supermix for Probes (No dUTP) (BIORAD)
ddPCR™ CNV Assay, Validated (HEX) primer/probe (BIORAD)
Droplet Generation Oil for Probes (BIORAD)
Ethidium bromide solution (Sigma-Aldrich)
Glycogen (Life Technologies)
LightCycler® 486 SYBR Green I Master (Roche)
LINE 97/247 primers (Invitrogen™)
NORGEN Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit (Norgen Biotek)
Nuclease-free water (Life Technologies)
Power SYBR™ Green PCR Master Mix (Applied biosystems)
PowerUp™ SYBR™ Green Master Mix (Applied biosystems)
QIAamp® Circulating Nucleic Acid (Qiagen)
Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen)
SsoAdvanced™ Universal SYBR® Green Supermix (BIORAD)
TBE buffer (Tris-borate-EDTA) Sigma-Aldrich)
Uracil-DNA Glycosylase (Life Technologies)
ΦX174 DNA-HaeIII Digest marker (New England Biolabs)

Contents of each kit is listed before the described protocol in the chapter CfDNA isolation and CfDNA quantification by Quant-iT™ PicoGreen™ dsDNA Assay Kit.

4.2. Optimisation part

4.2.1. Patients and sample collection

4.2.1.1. Samples used in optimisation part

Samples used for optimisation purposes were samples of DNA from two breast cancer cell lines, MCF-7 and MDA-MB-231 (American Type Culture Collection). Work with the cell lines was done by Ing. Marie Ehrlichová (Department of Toxicogenomics, SZU), DNA was isolated by phenol-chloroform extraction method (FCH) (Topić and Gluhak, 1991) and by AllPrep DNA/RNA/Protein Mini Kit (APK) according to manufacturer's protocol by Stanislav Horský (Department of Toxicogenomics, SZU). Quality and concentration of DNA were assessed using Quant-iT PicoGreen dsDNA Assay Kit and NanoDrop2000 (Thermo Scientific) according to the manufacturer's protocol. In the first step of the optimisation part, DNA samples isolated by both isolation methods were used.

Other samples used in optimisation part for cfDNA isolation and quantification methods were plasma (P) and serum (S) samples obtained from four women with unknown medical history. On the day of surgery, patients' peripheral blood samples were collected into serum tubes and EDTA-containing plasma tubes. Samples were centrifuged at 2 500 g for 5 min at 4°C and plasma/serum portion were recentrifuged at 2 500 g for 5 min at 4°C to obtain cell-free samples. Samples were aliquoted and stored at -80°C for further analyses.

4.2.2. CfDNA isolation

For this study, NORGEN Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit and QIAamp® Circulating Nucleic Acid (QIAamp CNA kit) were used and their efficiency compared for plasma and serum samples. Both isolation kits are based on similar four standard steps - lysis, binding, wash and elution. In addition, QIAamp CNA kit includes small carrier RNA. The buffer with carrier RNA, together with proteinase K, ensures and enhances effective release of nucleic acids from proteins and vesicles and their binding to spin column membrane. Furthermore, carrier RNA decreases the chance of RNA degradation by potentially active RNases. Also, both kits contain spin columns that bind even short fragments of DNA.

At the beginning of the isolation process, P and S samples were centrifuged to remove cryoprecipitates (16 000g, 5 min, 4°C). Moreover, we have added a small amount of glycogen

to half of our test samples during a certain point of isolations as it is generally used as carrier molecule and co-precipitant to optimize the recovery of small amounts of nucleic acids. The centrifugation step before isolation and adding of the glycogen is not included in either Norgen or Qiagen standard isolation protocol.

CfDNA isolation was carried out in laminar airflow cabinet and all used plastic equipment was sterile and RNase and DNase free.

4.2.2.1. Isolation using QIAamp® Circulating Nucleic Acid kit:

Contents:
QIAGEN® Mini columns 50
Collection Tubes (2.0 ml)
Elution Tubes (1.5 ml)
Buffer ACL 220 ml
Buffer ACB (concentrate) 300 ml
Buffer ACW1 (concentrate) 19 ml
Buffer ACW2 (concentrate) 13 ml
Buffer AVE (purple caps) 5 x 2 ml
QIAGEN Proteinase K 4 x 7 ml
Carrier RNA 310 µg

Before we started the isolation, isopropanol or ethanol were added to concentrated buffers ACB, ACW1 and ACW2 to obtain working solutions according to manufacturer's protocol. Additionally, lyophilised carrier RNA had to be dissolved in Buffer AVE. The correct amount of carrier RNA in Buffer AVE with Buffer ACL was calculated following the manufacturer's protocol – 5.6 µl of Carrier RNA in Buffer AVE was added into 0.9 ml of Buffer ACL per one 1 ml sample of plasma or serum to obtain concentration 0.2 µg/µl. Carrier RNA in AVE Buffer was then divided into separate 20 µl aliquots and stored at -20°C to avoid thawing of frozen carrier RNA more than three times, due to manufacturer's recommendations.

After this preparation, serum and plasma samples were centrifuged to remove cryoprecipitates (16 000 g, 5 min, 4°C) as mentioned above. Then we pipetted 1 ml of each sample into 2 ml centrifuge tube containing 100 µl of Proteinase K. Subsequently, 0.8 ml of Buffer ACL containing carrier RNA was added, pulse-vortexed and immediately incubated

for 30 min at 60°C. After this step, the lysate was transferred into 5 ml tube containing 1.8 ml of binding Buffer ACB. During this part, we added 1 µl of glycogen (20 µg/µl) to the corresponding mixture of samples. All mixtures (with and without glycogen) were then pulse-vortexed and incubated on ice for 5 min. The mixture was then transferred into spin column, centrifuged (3 300 g, 1 min, 24°C) and the eluate was removed. This step was repeated until all the mixture was spun through the spin column.

Since cfDNA remains bound to the silica membrane, other impurities are removed by adding wash buffers into solution during the following wash steps with 600 µl of Buffer ACW1, 750 µl of Buffer ACW2 and 750 µl of absolute ethanol (3 300 g, 1 min, 24°C). After these steps, samples were spun at full speed (20 000 g, 3 min, 24°C) and the spin columns were then incubated at 56°C for 10 min in new collection tubes with open lid to dry the membrane completely. The spin columns were placed into new elution tubes and 50 µl of elution Buffer AVE was applied into the centre of the silica membrane of the column and incubated for 3 min in room temperature. After subsequent centrifugation (20 000 g, 1 min, 24°C), the eluate contained nucleic acids.

4.2.2.2. Isolation using NORGEN Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit:

Contents:
Binding Buffer B 40 ml
Proteinase K 0.6 ml
Wash Solution A 18 ml
Elution Buffer B 8 ml
Mini Spin Columns 50
Collection Tubes
Elution tubes (1.7 mL)

Before isolation, appropriate amount of ethanol was added to concentrate Wash Solution A according to manufacturer's protocol. cfDNA was isolated from samples of 500 µl volume in contrast to QIAamp kit. Samples were centrifuged (16 000 g, 5 min, 4°C). Subsequently, 12 µl of vortexed Proteinase K was added into each sample, then shortly vortexed and incubated for 10 min at 55°C. After this step, 1 ml of Binding Buffer B along with 1 µl of glycogen (20 µg/µl) was added to half of samples and vortexed. Afterwards, the

mixture was transferred into spin columns and centrifuged (3 300 g, 2 min, 24°C) and the eluate was removed. This step was repeated until the whole mixture was centrifuged through the spin columns. Following washing part included applying 600 µl of Wash Solution A to the spin column and centrifugation (3300 g, 1 min, 24°C). Washing step had to be repeated twice. Samples were then spun (13 000 g, 2 min, 24°C), transferred into new elution tubes and 50 µl of Elution Buffer B was applied to the centre of the column and incubated for 2 min at room temperature. Spin columns were then centrifuged two times (400 g, 1 min, then 5 800 g, 2 min, 24°C). Eluted buffer with cfDNA was transferred back into spin columns, and the last step has been repeated one more time for maximum recovery of cfDNA, according to manufacturer's recommendation.

The main difference between the two isolation kits was utilisation of carrier RNA by the QIAamp CNA kit. Moreover, QIAamp CNA kit recommends three wash steps, the last of which suggests adding ethanol, while Norgen kit contains only two wash steps using one wash buffer. All isolated samples were afterwards stored at -20°C.

4.2.3. CfDNA quantification by Quant-iT™ PicoGreen™ dsDNA Assay Kit

Contents:
Quant-iT PicoGreen dsDNA Reagent (Component A)
20x TE Buffer, DNase-free (Component B)
Lambda DNA standard (Component C)

Quant-iT™ PicoGreen™ is a fluorescent nucleic acid stain for dsDNA measurement. This assay allows measuring in a range from 25 pg/ml to 1 µg/ml using a standard spectrofluorometer. Sensitivity and selectivity for dsDNA (distinguishing it from RNA and ssDNA are the main advantages of the method which also minimizes the influence of contaminants such as proteins, ethanol etc.

The standard curve was made with bacteriophage lambda DNA. The points of the standard curve were adjusted to "low-range assay" in order to effectively measure usual low concentrations of cfDNA. Since the approximate amount of isolated cfDNA occurs in a range from 1 to 100 ng/ml of plasma, it was decided to use five-point standard curve from 0.2 ng/ml to 100 ng/ml according to the manufacturer´s protocol (tab.5).

1x TE (µl)	Lambda dsDNA STD (µl)	PicoGreen Reagent Working Solution (µl)	Concentration of DNA (ng/ml)
686	14	100	**1000 ng/ml**
450	50	100	**100 ng/ml**
400	100	100	**25 ng/ml**
400	100	100	**5 ng/ml**
400	100	100	**1 ng/ml**
400	100	100	**0.2 ng/ml**
400	0	100	**0 ng/ml**

Table 6. The preparation of low-range standard curve.

At the beginning of the measurement, 1x TE buffer was prepared by dilution of the appropriate amount of 20x TE buffer in Nuclease-free water (Life Technologies) and 200x solution of Quant-iT PicoGreen dsDNA Reagent Working Solution was diluted with 1x TE buffer according to a number of measured samples and protocol. A standard curve was prepared by making a dilution series of corresponding concentrations using Lambda DNA standard as written in table 3. The volume of 100 µl of each diluted standard solution was then transferred into 96-well plate.

Measured samples of isolated DNA were diluted directly in the wells of the plate by transferring 1 µl of each sample followed by 99 µl of 1x TE buffer. In the last step, 100 µl of PicoGreen Reagent Working Solution was applied into all wells (containing standard and sample DNA) to reach a total volume of 200 µl of each reaction. All samples and points of the standard curve were measured in duplicates.

The measurement was accomplished using a spectrofluorometric plate reader Infinite 200 (TECAN) connected with software i-Control 1.3 (TECAN). Specific parameters of reading the fluorescence were set to: excitation λ = 480 nm, emission λ = 520 nm, number of reads, 5.

4.2.4. Quantification method quantitative real-time PCR

Another mentioned aim was to optimize the quantitative real-time polymerase chain reaction (qPCR) method for quantification of low levels of cfDNA and subsequent analysis of cfDNA integrity. qPCR is a reliable assay for absolute or relative DNA quantification. It measures the DNA in real-time (after each amplification cycle), by means of fluorescent dye that binds to dsDNA non-specifically (e.g., SYBR Green) or by sequence-specific oligonucleotide probe (e.g., TaqMan).

In the first part of optimisation, we used ALU-115/247 primers (Umetani et al., 2006) with SYBR Green detection according to a published protocol (see next parts *4.2.4.1. Selection of DNA standard and range of standard curve* and *4.2.4.2 Selection of primers*).

Due to poor results obtained with this assay (*5 Results*), other pairs of primers specific for repetitive and non-repetitive sequences (*4.2.4.2. Selection of primers*), different SYBR green master mixes (*4.2.4.3. Selection of DNA stain*) and qPCR protocols were tested. PCR reactions were performed on RotorGene 6000 cycler (Qiagen) and analysed using Rotor-Gene Q Software 2.3 (Qiagen). Non-template (NTC) control was included in every PCR plate.

During qPCR optimisation we focused on several factors:

- PCR efficiency - the rate, at which a PCR amplicon is generated, calculated as a percentage value. If the efficiency of a PCR reaction is 100%, it means that the amplicon doubles its amount during an exponential phase. Optimal efficiency of PCR reaction is 90-110%.
- Linearity – R^2 statistic value for standard curve, optimal R^2 should be ≥ 0.98.
- Specificity – melt curve analysis was used in order to identify the unwanted non-specific PCR products. Melt curve analysis is post-PCR process, which is based on the slow rising of temperature in order to denature the strands of DNA amplicons to ssDNA. As the temperature increases, fluorescent dye is dissociated from dsDNA and the fluorescent signal starts to decrease. The temperature in which the signal drops depends on amplicon length and nucleotide pairing in the DNA strands.
- Sensitivity – the lowest concentration of the serial dilution of standard, where replicate reproducibility is high and linear.

4.2.4.1. Selection of DNA standard and range of standard curve

Appropriate DNA standard and range of DNA standard curve for quantification of cfDNA had to be selected. DNA from two cell lines isolated by two methods (see *4.2.1.1. Samples used in optimisation part*) was used for the construction of the standard curve. These samples were diluted to concentrations from 10 000 to 0.01 pg per reaction and measured by qPCR with ALU-115 primers for short 115 bp fragments.

STD/rxn (pg)
10000
1000
100
10
1
0.1
0.01

Samples: DNA isolated from cell lines MCF-7 (FCH, APK), MDA-MB-231 (FCH,APK)
Primers: ALU-115 (200nM)
Range of standard curve: 10000 – 0.01 pg/rxn
qPCR amplification mix compounds per sample (total volume of each reaction was 10 µl): 2.1 µl dH2O, 0.2 µl ALU-115 (10uM) forward primer, 0.2 µl ALU-115 (10uM) reverse primer, 5.0 µl LC486 SYBR Green I Master (Roche), 2.5 µl template DNA. This composition of qPCR master mix was used for all subsequent qPCR reactions.

qPCR cycling program:

hold	95°C - 10 min
40 cycles	95°C – 15s
	60°C – 60s
melt curve	from 60 to 99°C with reading every 1°C

After amplification, 10 µl of samples were mixed with 2 µl of 6x Loading Dye Solution and subsequently separated by electrophoresis on 3% agarose gel in 0.5x concentrated TBE buffer at 120V and current 100mA for 25 min. Utilised marker was 8 µl premixed size standard ΦX174 DNA Hae III Digest (6x ΦX174 DNA Hae III Digest and 6x Loading Dye Solution in Nuclease-free water). Samples were then stained in intercalating Ethidium bromide (10ng/ml) solution for 5 min and photographed by Odyssey® CLx Imaging System (LI-COR Biosciences).

4.2.4.2. Selection of primers

Firstly, three different concentrations of both ALU-115 and ALU-247 (Umetani et al., 2006) primers were measured using three annealing temperatures:

Samples: DNA isolated from MCF-7 cell line
Primers: ALU-115, ALU-247
Primer concentrations: 50nM/200nM/500nM
Range of MCF-7 standard curve: 100 – 0.01 pg/rxn.
qPCR program:

hold	95°C - 10 min
40 cycles	95°C – 15s
	57°C/60°C/63°C – 60s
melt curve	from 60 to 99°C with reading every 1°C

Concentration of 200nM was used for subsequent reactions. Afterwards, different pairs of primers were tested: primers for beta-actin gene (ACTB 117/382), another pair of primers for ALU repetitive DNA elements (ALU 111/260) and primers for LINE repetitive DNA elements (LINE 97/266). Sequences of the primers are listed in the table 6.

Primers	Forward sequence 5'-3'	Reverse sequence 5'-3'	Product	Source
ALU-115	CCTGAGGTCAGGAGTTCGAG	CCCGAGTAGCTGGGATTACA	115 bp	*Umetani et al., 2006*
ALU-247	GTGGCTCACGCCTGTAATC	CAGGCTGGAGTGCAGTGG	247 bp	
ACTB-117	CTGGCACCACACCTTCTACA	CCACTCACCTGGGTCATCTT	117 bp	designed for this project
ACTB-382	CTGGCACCACACCTTCTACA	GCTTTACACCAGCCTCATGG	382 bp	
ALU-111	CTGGCCAACATGGTGAAAC	AGCGATTCTCCTGCCTCAG	111 bp	*Madha-van et al., 2014*
ALU-260	ACGCCTGTAATCCCAGCA	CGGAGTCTCGCTCTGTCG	260 bp	
LINE-97	TGGCACATATACACCATGGAA	TGAGAATGATGGTTTCCAATTTC	97 bp	
LINE-266	ACTTGGAACCAACCCAAATG	CACCACAGTCCCCAGAGTG	266 bp	

Table 6. List of utilised primers

In the beginning, all of the primers were measured using MCF-7 standard curve with range 100 – 0.01 pg/rxn, except for PCR reactions with ACTB primers – in this case, the range was adjusted to 10 – 0.001 ng/rxn. Afterwards, test samples P464, P470, P281 and P282 were measured (see *4.2.1.1. Samples used in optimisation part*).

Samples: DNA isolated from MCF-7 cell line, and from serum (S) and plasma (P) samples P464, P470, P281, P282 (see *4.2.1.1. Samples used in optimisation part*)

qPCR programs:

hold	95°C – 10 min
35 cycles	95°C – 15s
	63°C/65°C – 60s
	72°C – 15s
melt curve	from 60 to 99°C with reading every 1°C

4.2.4.3. Selection of DNA stain

Three different master mixes were tested: LightCycler® 486 SYBR Green I Master (Roche), Power SYBR™ Green PCR Master Mix, PowerUp™ SYBR™ Green Master Mix (both from Applied biosystems), and SsoAdvanced™ Universal SYBR® Green Supermix (BIORAD) were measured on two sets of primers, ACTB and LINE primers. LightCycler® 486 SYBR Green I Master, utilised for all previous qPCR reactions, is a stain usually utilised in the laboratory of supervisor. Moreover, because we still got positive amplification in NTC in reactions containing primers for repetitive sequences (see chapter *Results*, tab x.), we added Uracil N-glycosylase (UNG, Life Technologies) to PCR reactions to prevent carryover contamination (1 U/µl therefore 0.1 µl of UNG per 10 µl reaction). SYBR Green Master Mixes used in our qPCR reactions contain dUTP nucleotides which are randomly incorporated in new PCR products during amplification. Uracil N-glycosylase is an enzyme which cleaves N-glycosylic bond between uracil and saccharide in ssDNA and dsDNA, which ensures cleavage and degradation of possible DNA contamination from previous qPCR reactions. PowerUp™ SYBR™ Green Master Mix (Applied biosystems) already contains UNG.

The UNG treatment is performed at 50°C for 2 min at the onset of the cycling program. Because optimal results from *selection of primers* part were performed using ACTB and LINE primers, we used them also for the selection of appropriate stain.

Samples: DNA from MCF-7 cell line, and from P281P and P281S

Primers: ACTB-117, ACTB-382, LINE-97, LINE-266

qPCR program:

hold 1	50°C – 2 min
hold 2	95°C – 10 min
40 cycles	95°C – 10s
	63°C – 60s
	72°C – 15s
melt curve	from 60 to 99°C with reading every 1°C

4.3. Measurement part

4.3.1. Samples used in the measurement part

In the measurement part, peripheral blood samples from patients with breast, ovarian, colorectal, and pancreatic carcinomas, from patients with benign diseases of breast and ovary, and samples from healthy volunteers were utilised. Breast carcinoma patients were collected in The Faculty Hospital Motol and in The Institute for the Care for Mother and Child (Prague). Patients with ovarian and colorectal carcinoma were collected in The University Hospital Pilsen (Pilsen), and pancreatic carcinoma patients in The University Hospital Olomouc (Olomouc). Blood samples from healthy volunteers were collected in Olomouc. Personal and clinical characteristics of studied patients are described in the tables 5 and 6.

Plasma and serum samples were separated from whole blood samples as described in chapter *4.2.1.1. Samples used in optimisation part*.

The study was approved by the Ethical Commission of the National Institute of Public Health in Prague. All patients were informed about the study aims and those who agreed and signed an informed consent participated in the study. Clinical data are summarized in the table 5. and 6.

Disease	N	Sex	Median age (range)	Diagnosis (N)
Breast	25	25 Female	60 (30-77)	Benign disease (5) Non-invasive breast carcinoma (5): *Ductal carcinoma in situ (5)* Invasive breast carcinoma (10): *Invasive ductal carcinoma (7)* *Invasive lobular carcinoma (2)* *Mixed type (1)*
Ovarian	14	14 Female	65 (38-89)	Benign disease (4) Ovarian carcinoma (9): *Serous carcinoma (4)* *Mucinous carcinoma (2)* *Endometrioid carcinoma (2)* *Clear cell carcinoma (1)*
Colorectal	20	9 Female 11 Male	66 (46-82)	Colorectal carcinoma (20): *Colon carcinoma (10)* *Rectal carcinoma (7)* *Rectosigmoid carcinoma (3)*
Pancreatic	10	6 Female 4 Male	64 (52-74)	Pancreatic adenocarcinoma (10)
Healthy controls	18	10 Female 8 Male	53 (39-61)	No cancer diagnosis in personal history.

Table 5. Patients and controls involved in the study.

N = number of cases.

Clinical characteristics	Breast N = 20	Ovarian N = 9	Pancreatic N = 10	Colorectal N = 20
Stage				
0	5	0	0	0
I	5	3	1	5
II	5	1	4	5
III	5	5	0	5
IV	0	0	5	5
pT				
pTis	5	Not evaluated	0	0
pT1	11		1	0
pT2	4		0	5
pT3	0		8	13
pT4	0		1	2
pN				
pN0	10	Not evaluated	2	10
pN1	5		8	5
pN2	5		0	5
Distant metastasis				
cM0	20	4	5	15
cM1	0	0	5	5
unknown	0	5	0	0
Grade				
1	7	1	Not evaluated	5
2	9	1		13
3	4	7		2

Table 6. Clinical characteristics of carcinoma patients

4.3.2. Isolation and quantification methods used in measurement part

The particular methods based on results from the optimisation part were utilised (see *4 Results, 4.1 Optimisation part*). Plasma samples were isolated using QIAamp CNA kit, cfDNA concentration was quantified by PicoGreen assay using low-range protocol and by qPCR using LINE-97 primers. cfDNA integrity was also assessed by set of LINE-97/LINE-266 primers.

qPCR amplification mix compounds per sample (total volume of each reaction was 10 µl):
2 µl dH2O, 0.2 µl LINE-97/LINE-266 (10 µM) forward primer, 0.2 µl LINE-97/LINE-266 (10 µM) reverse primer, 5.0 µl LC486 SYBR Green I Master (Roche), 0.1 µl UNG, 2.5 µl template DNA.

qPCR program used for all samples:

hold1	50°C – 2 min
hold2	95°C – 10 min
40 cycles	95°C – 15s
	63°C – 60s
melt curve	from 60 to 99°C with reading every 1°C

4.3.3. Droplet digital PCR

Droplet digital PCR (ddPCR) is a modern approach based on PCR technology. The sample is firstly compartmentalized into thousands of water-oil droplets and then PCR-amplified in each droplet (Hudecova, 2015). Of total number of samples, 44 samples were selected and measured due to insufficiency of chemicals required for all samples (tab. 7).

Disease	Stage	N
Male controls		4
Female controls		5
Breast cancer	III	5
Ovarian cancer	III	5
Colorectal cancer	I	5
Colorectal cancer	II	5
Colorectal cancer	III	5
Colorectal cancer	IV	5
Pancreatic cancer	IV	5

Table 7. List of samples used in ddPCR assay

The concentration of cfDNA was established using primers with probe specific for RNaseP gene by copy number assay and the QX200™ Droplet Digital™ PCR System (BioRad), as RNAseP is rarely mutated gene or affected by copy number alterations (Earl et al., 2015). In ddPCR experiment, the sample is randomly distributed into discrete droplets, thus some droplets contain no DNA template and others contain one or more template copies. After PCR amplification, each droplet is analysed to determined fraction of target positive droplets and the target DNA template concentration is determined using Poisson statistics (Hudson et al., 2011).

First step of the measurement was preparation of a diluent buffer containing a restriction enzyme following table 8, using 4 units of enzyme *HaeIII* per reaction

as recommended by manufacturer. Digestion of the DNA was carried out directly in the ddPCR reactions and ensures even distribution of the fragments into droplets, reducing sample viscosity, help to separate tandem gene copies and improve template accessibility (ddPCR™ Copy Number Variation Assay manual, BioRad).

	ul for 4U/rxn
NFW	0.54
CutSmart buffer (10×)	0.06
***HaeIII* 10U/ul**	0.4

Table 8. Preparation of diluent buffer for restriction enzyme.

All reactions were set up in room temperature. Buffer containing the enzyme was then added to prepared master mix according to table 9.

Component	**µl/rxn**	**Final Concentration**
2x ddPCR Supermix for Probes (No dUTP)	11	1×
20x primers/probe (HEX)	1	1×
Restriction enzyme, diluted	1	4 U/rxn
NFW	7	
Total volume per reaction	21	

Table 9. Preparation of the master mix.

The master mix was then vortexed, centrifuged and transferred into 0.2 ml tube. Subsequently, 1 µl of cfDNA sample was added to final volume of the reaction 22 µl, vortexed, centrifuged and incubated in room temperature for 3 min. Next step was loading 20 µl of each sample into specific sample wells of DG8 ™ Cartridge designed for Droplet Generator. Next, 70 µl of Droplet Generation Oil was added into oil wells, the cartridge was covered with the rubber cover and transferred into Droplet Generator. After droplet generation, droplets present in sample wells were transferred into 96-well plate, which was then sealed with aluminium foil using PCR Plate sealer and transferred into the cycler. Cycling was conducted following program described in table 10. Finally, after PCR reactions were performed, droplets were read using QX200 Droplet Reader. Data were acquired and analysed using QuantaSoft™ Software.

Cycling Step	Temp., °C	Time	Ramp Rate	Number of Cycles
Enzyme activation	95	10 min		1
Denaturation	94	30 s	2°C/s	40
Annealing/extension	60	1 min		
Enzyme deactivation	98	10 min		1
Hold	4	Infinite	1°C/s	1

Table 10. The cycling program of the ddPCR

4.4. Statistical analysis

The normality of the results was checked by the Shapiro-Wilk test. Raw data were not normally distributed, thus non-parametric tests were used for further analysis. Results obtained by comparing two or more groups of data were assessed using the Mann-Whitney and the Kruskal-Wallis statistical tests. Correlations between groups were determined by the Spearman non-parametric test. A p value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS v16.0 software (SPSS Inc., Chicago, IL).

5 Results

5.1. Optimisation part

5.1.1. CfDNA Isolation

The results of testing the isolation kits revealed considerable difference between the obtained yields (fig.13, tab.11). Glycogen was expected to aid the recovery of the yield; however, no significant effect on the extracted DNA was observed (samples P464 and P470) (fig.14). Larger volume of isolated cfDNA was obtained by QIAamp CNA kit. Furthermore, higher amount of cfDNA was isolated from serum, independently of isolation kit (tab.11).

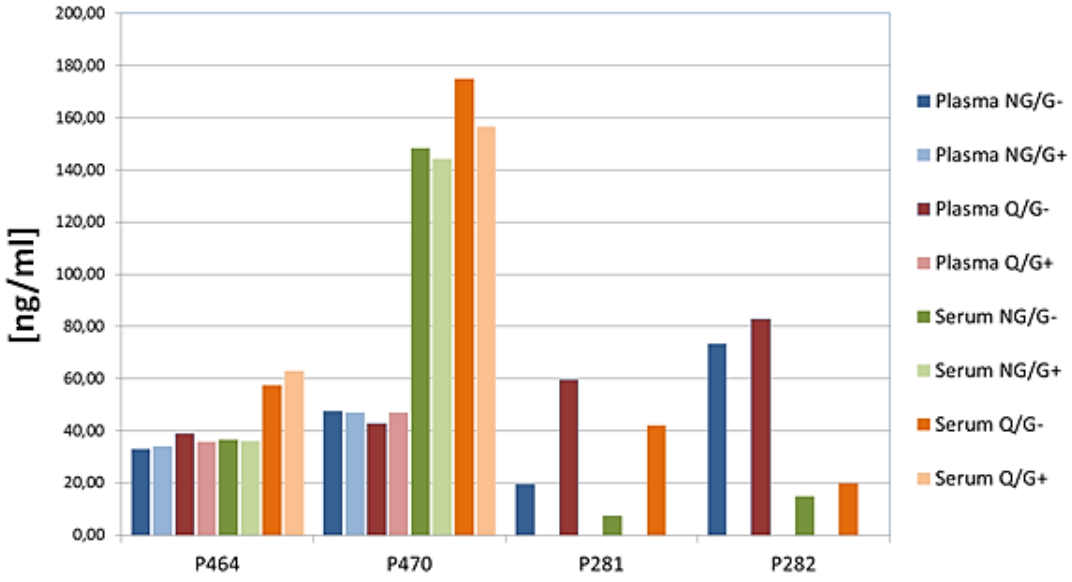

Figure 14. **Comparison of cfDNA yields isolated by different isolation kits.** NG = NORGEN P/S purification mini kit; Q = QIAamp CNA kit; G+ = added glycogen, G- = no added glycogen. CfDNA concentration was estimated by PicoGreen assay.

	Plasma (ng/ml)	Serum (ng/ml)
NORGEN P/S purification mini kit	43.3 ± 23.1	51.9 ± 65.4
QIAamp CNA kit	55.9 ± 20.0	73.6 ± 69.3

Table 11. The average amounts of DNA isolated from samples P464, P470, P281 and P282 using both kits without added glycogen.

5.1.2. Quantification methods

PicoGreen assay was optimized for low-range assay to detect concentrations as low as 25 pg/ml. Measured concentrations of test sample P464 and the difference between measurements performed using high-range and low-range protocol are apparent from table 12.

Sample: P464	Isolation kit	High-range protocol (ng/ml)	Low-range protocol (ng/ml)
PLASMA	Norgen G-	62.0	32.9
	Norgen G+	13.2	34.2
	Qiagen G-	44.6	38.8
	Qiagen G+	10.4	35.8
SERUM	Norgen G-	15.5	36.7
	Norgen G+	9.4	35.9
	Qiagen G-	41.9	57.4
	Qiagen G+	48.1	62.9

Table 12. Yields of cfDNA obtained by both isolation kits. Notes: G+: added glycogen; G- : without added glycogen; Norgen: Norgen P/S purification kit; Qiagen: QIAamp CNA kit;

During qPCR optimisation were tested several variations of qPCR programs in order to achieve the highest possible specificity and sensitivity of the reactions. Quantity and quality of isolated DNA from MCF-7 and MDA-MB-231 cell lines by FCH method were slightly higher compared to DNA isolated from these cell lines by APK, thus DNA from MCF-7 (FCH) was used as the standard in qPCR reactions.

The first tested range of standard curve 10 000 – 0.01 pg/rxn markedly decreased the R^2 value of the reactions with ALU-115/247 primers. Moreover, very low concentration of the last point of the curve was similar to positive signal which was detected in the NTC – average Ct cycle of the last point was 23.3, whereas for NTC it was 26.2 using ALU-115.

The optimal values of R^2 and efficiency were acquired using the range from 1 to 0.1 pg/rxn of the standard curve; therefore, we decided to set this range for the following qPCR reactions. No significant effect on efficiency between two-step and three-step PCR program was observed.

The efficiency value in reactions containing ALU-115 and ALU-247 primers were near 99 % ($R^2 \geq 0.98$), however, the melt-curve analysis revealed non-specific products in PCR in the NTC (fig.15). In order to confirm these results we analysed the qPCR products on electrophoretic agarose gel, where the NTC was positive as well, mainly for ALU-115 (fig.16).

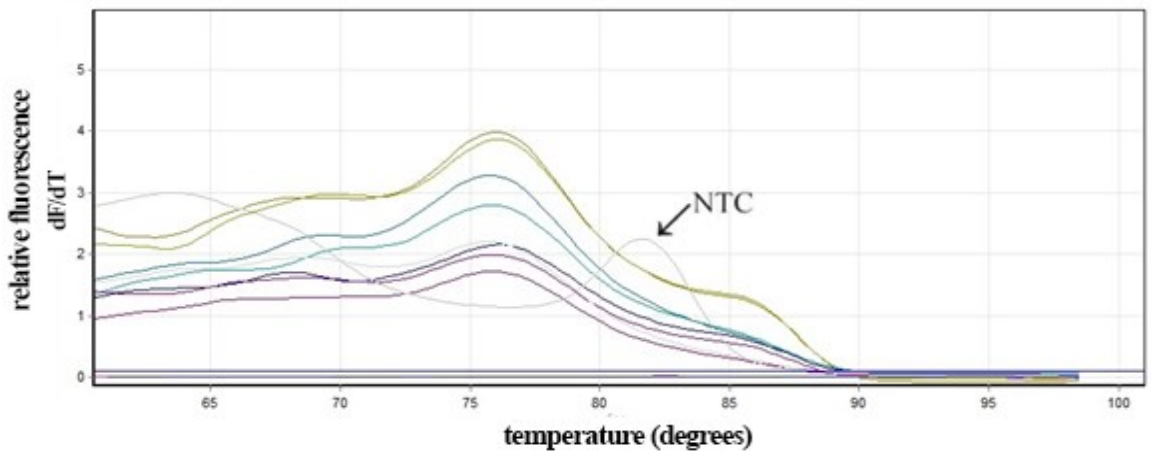

Figure 15. Melt-curve analysis of reaction with MCF-7 standard. ALU-115 primers, annealing temperature 63°C. NTC: non-template control.

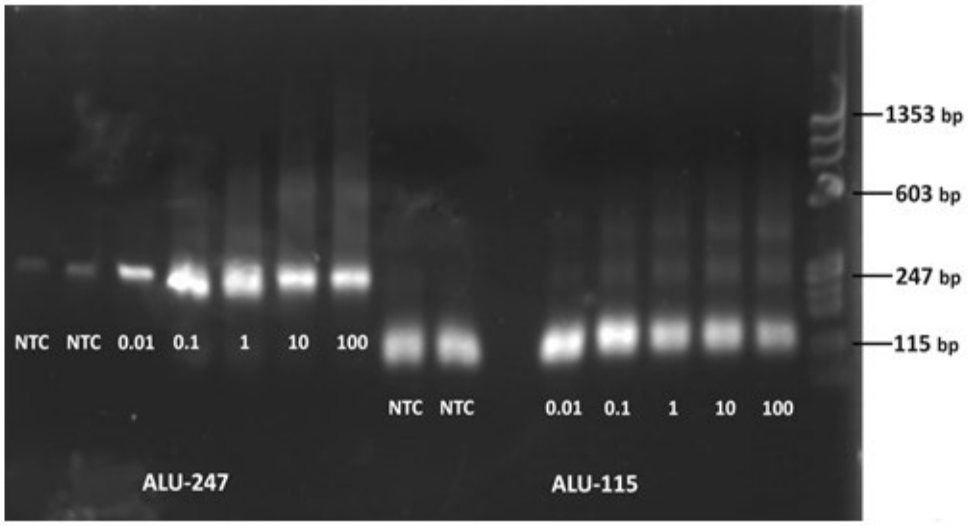

Figure 16. Products of qPCR reaction using ALU-115 and ALU-247 primers. Standard DNA of concentrations 100 – 0.01 pg/rxn (40 – 0.04 pg/µl), described below each sample. Amplification products were present in all non-template controls.

Following previous results that implied the presence of non-specific products in PCR reactions using ALU-115/247 primers, new pairs of primers for short and long fragments were tested (table 4, chapter 4.2.4.2. *Selection of primers*). Firstly, all primers were analysed on MCF-7 standard curve. Many of them worked differently depending on specific conditions (tab.13). The correlation coefficient and efficiency of the reactions were again near 100 %, however, the melt curve analysis of the qPCR still showed non-specific products, mainly in reactions with the new ALU primers.

The most promising results were observed in the reactions using primers for ACTB-117, and LINE repetitive DNA elements. The R^2 value and efficiency of the reactions were near to 100 %, thus the primers were subsequently utilised in an extra qPCR reaction using 65°C annealing temperature. Melt curve analysis of the reactions with ACTB primers revealed a large amount of non-specificity independently of annealing temperature (fig. 17). On the other hand, the desired single peak in PCR samples was observed in melt curve of qPCR reactions with LINE, which confirmed the specificity of the primers.

Yet NTCs of the reactions with LINE primers were still positive despite adding UNG treatment to the protocol and testing of 4 different qPCR master mixes. However, non-specific products in NTCs occurred in latter Ct cycle in comparison to the last point of the standard curve: the range of Ct cycles of standard curve were 11 – 25 Ct, while Ct of the NTC was 29 for LINE-97; Ct of standard curve was 8 – 23, while Ct of the NTC was 31 for LINE-266, which was therefore set as a limit of quantification for LINE primers. All reactions conducted under specific conditions and obtained results are listed in the table 13.

Primers	Annealing temperature	Efficiency	R^2 value	No-template control	Non-specific products in melt curve analysis
ACTB-117	57°C	1.12	0.98	×	✓✓
	60°C	1.15	0.99	×	✓
	63°C	1.06	0.97	×	✓
	65°C	0.82	0.87	✓	✓
ACTB-382	57°C	1.16	0.98	×	✓✓
	60°C	1.03	0.97	×	✓
	63°C	0.91	0.96	×	✓
	65°C	0.91	0.97	×	✓✓
ALU-111	57°C	0.94	1.00	✓	✓✓
	60°C	1.00	1.00	✓	✓✓
	63°C	0.92	1.00	✓	✓✓
ALU-260	57°C	0.84	0.99	✓	✓
	60°C	0.96	0.93	✓	✓✓
	63°C	0.79	0.99	✓	✓✓
LINE-97	57°C	0.93	0.99	✓	×
	60°C	1.04	0.99	✓	×
	63°C	0.94	0.99	✓	×
	65°C	0.81	0.99	✓	×
LINE-266	57°C	0.93	1.00	✓	×
	60°C	1.09	0.98	✓	×
	63°C	0.94	1.00	✓	×
	65°C	0.86	0.99	✓	×

Table 13. Results obtained in qPCR reactions with different conditions.

Notes: Non-template control (NTC): (×) = products were not present in NTC; (✓) = products were present in NTC. Non-specific products in melt curve analysis: (×) = non-specific products were not present in PCR reaction; (✓) = non-specific products were present in PCR reaction; (✓✓) = high amount of nonspecific products was present in PCR reaction.

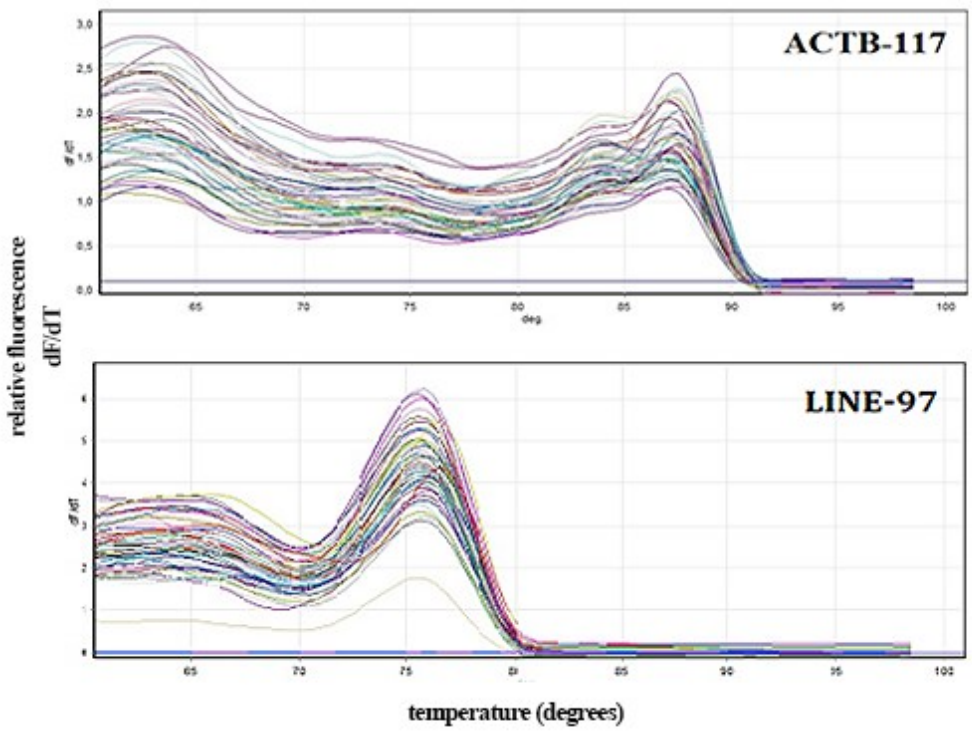

Figure 17. Comparison of melt curves between qPCR reaction using ACTB-117 and LINE-97 primers. Annealing temperature was set to 63°C, primer concentrations were 200 nM, samples: P464, P470, P281 and P282.

As a result of the qPCR optimisation part, LINE-97 and LINE-266 primers of 200nM concentration, LC486 SYBR Green I Master (Roche) and annealing temperature 63°C were selected as the most appropriate for purposes of this study. The DNA concentrations of most samples occurred in the range from 1 to 0.0001 ng/rxn of the standard curve, which confirmed correctly selected standard curve range (fig.18).

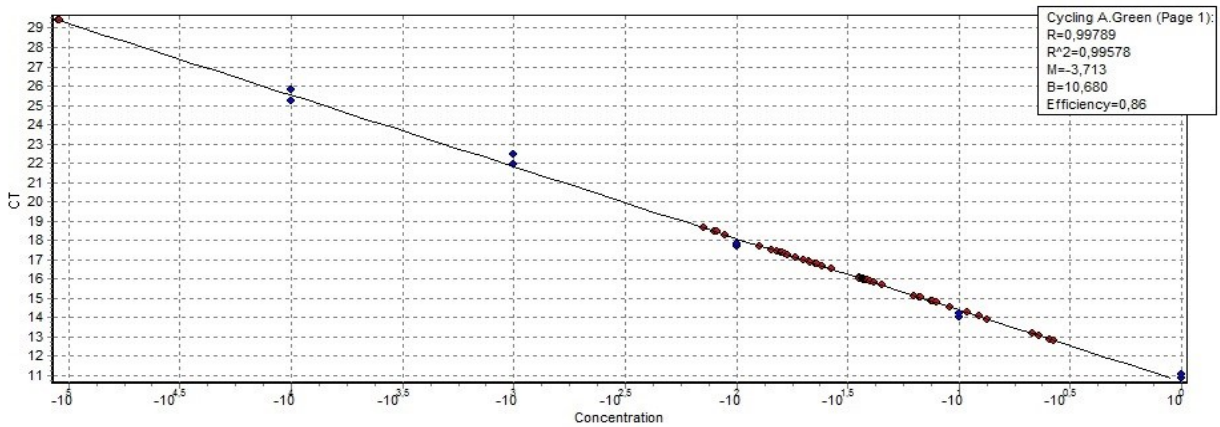

Figure 18. Points of standard curve in range 1 to 0.0001 ng/rxn (blue dots)
Samples P464, P470, P281, P282 of plasma and serum (red dots) using LINE-97.

5.2. Measurement part

In this part, cfDNA concentration in plasma samples of patients with tumour diagnoses and healthy controls was evaluated. Low range PicoGreen assay and qPCR with LINE-97/266 primers were performed following results from the optimisation part. The concentration of each sample was calculated as mean values of duplicate reactions. Moreover, cfDI values were calculated. Comparison of the results from Picogreen and qPCR LINE-97 assay is included in this chapter, as the cfDNA concentration was assessed by both of these methods.

5.2.1. Patients and clinical data

A total of 87 patients and healthy individuals were included in the study. More specifically, 25 patients with breast disease, 14 patients with ovarian disease, 20 patients with disease of colon or rectum, 10 patients with pancreatic disease, and 18 healthy controls were enrolled in the study. All personal and clinical data are summarized in the tables 5 and 6 (*4.3.1. Samples used in measurement part*).

5.2.2. Quantification of cfDNA

qPCR measurements were performed using LINE-97 primers on 69 plasma samples of patients with oncological diagnosis or benign cases and 18 samples of healthy individuals as well as measurement by PicoGreen low-range assay. Efficiency of all qPCR reactions was 93 - 100% and R^2 value 98 - 100%. Samples were measured in duplicates, with difference between duplicates being less than 0.5 of a Ct cycle. The Spearman test has revealed a strong correlation between concentration measured by PicoGreen assay and qPCR using LINE-97 ($p = 0.01$) (fig.19) However, concentrations assessed by PicoGreen with a mean of 35.7 ± 41.7 ng/ml were 3.8 times higher on average compared to mean LINE-97 cfDNA concentration (9.3 ± 11.2 ng/ml).

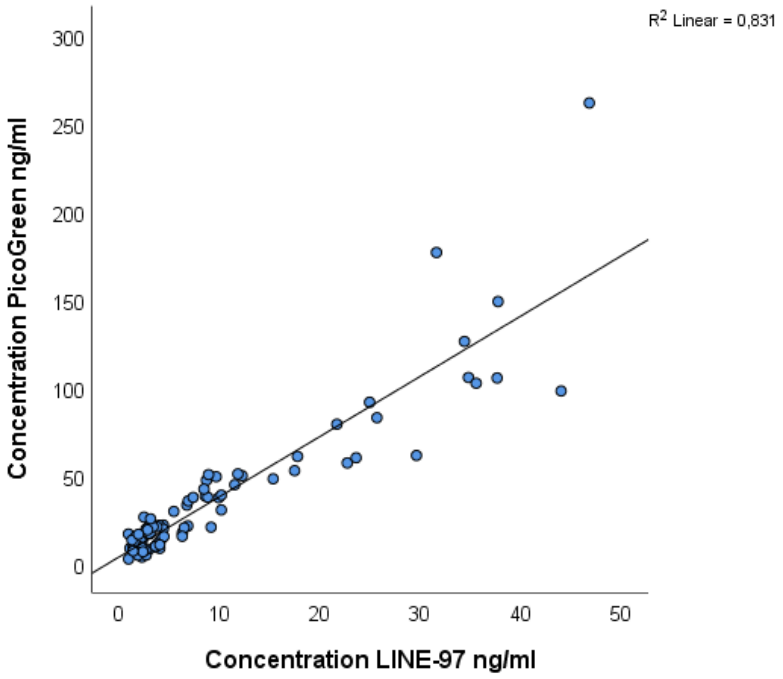

Figure 19. Correlation between all values measured by PicoGreen assay and qPCR using LINE-97, with correlation coefficient $R^2 = 0.831$.

Moreover, each group of samples seems to differ in relationship between cfDNA concentration and the type of particular disease (fig.20). The Kruskal-Wallis test confirmed significant differences between individual carcinoma types and healthy controls ($p < 0.001$), suggesting diverse results in individual categories. Figure 19 demonstrates similarity of PicoGreen and qPCR results obtained. Summary of analysed cfDNA concentrations is listed in table 15. Individual cancer groups are distinguished by colour - samples measured using qPCR LINE-97 are marked with blue colour markers, PicoGreen assay with green colour.

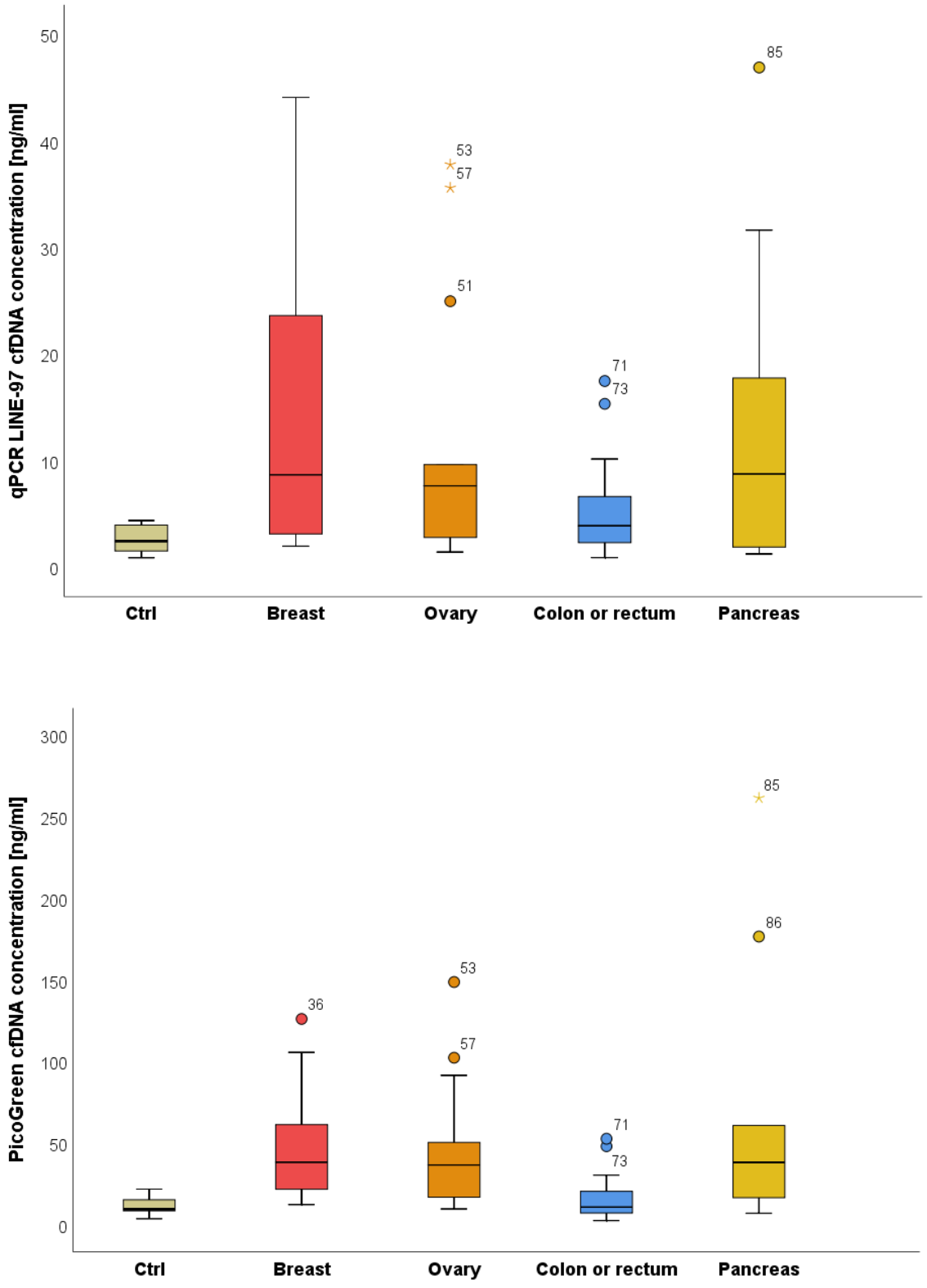

Figure 20. Boxplots with whisker-plots of cfDNA concentration of individual groups and quantification methods juxtaposed to each other. Boxes represent values within 25 – 75 percentiles with error bars and median. (○) = outliers; (*) = extreme outliers.

Breast cancer

For comparison of cfDNA concentration in patients with breast cancer female healthy controls only (n = 10) were used. Increased level of cfDNA concentration was observed in patients with invasive disease (SI - SIII, n = 15) compared to healthy controls independently on measurement method ($p < 0.001$) (fig.21, tab.15). Moreover, non-parametric Mann-Whitney test confirmed that cfDNA concentration was also elevated in patients with invasive disease (SI - SIII) (n=15) compared to group of patients with benign breast diagnoses (n = 15) ($p = 0.005$ for PicoGreen and $p = 0.011$ for qPCR).

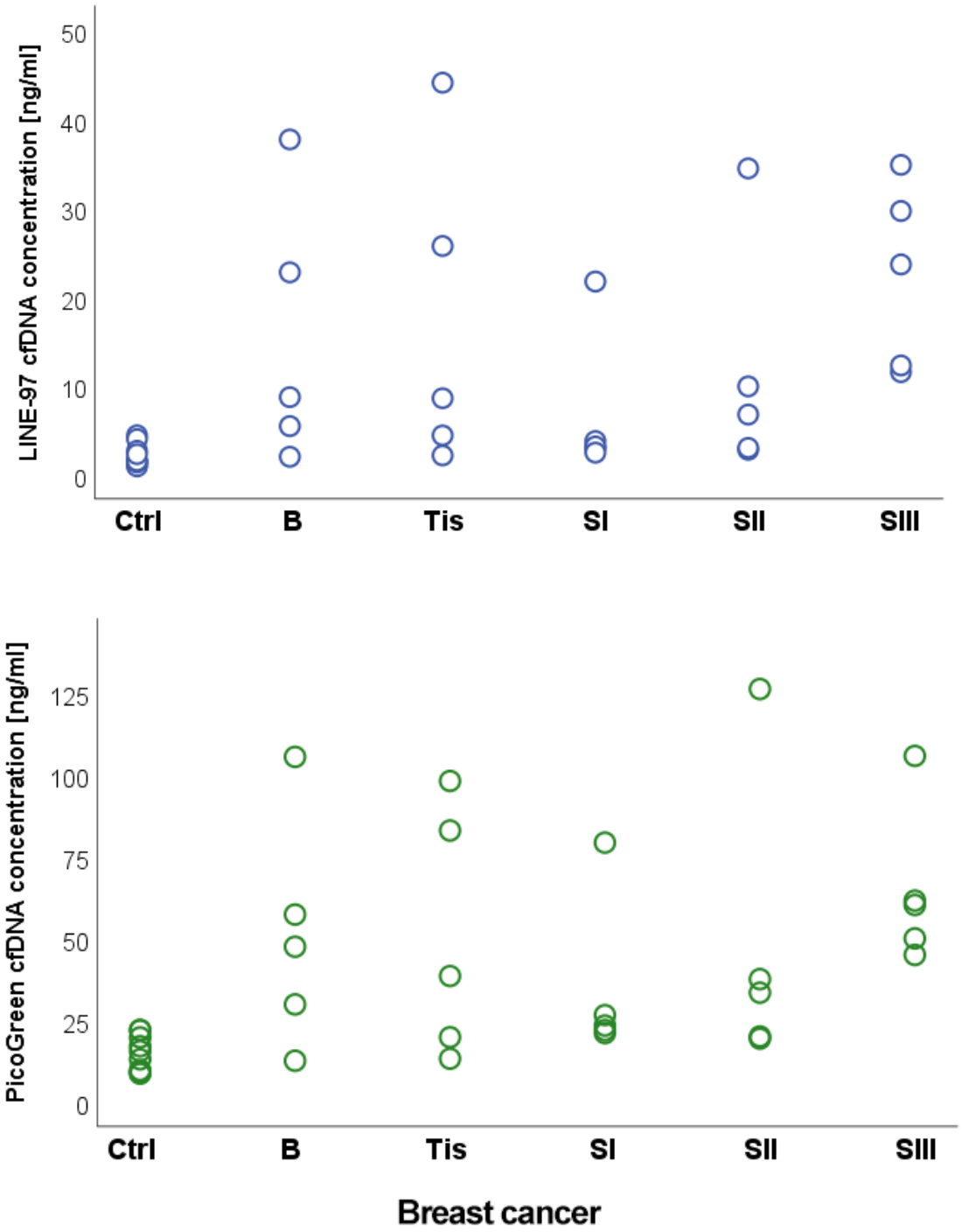

Figure 21. **Scatter plots of cfDNA concentrations measured by LINE-97 qPCR and PicoGreen assay in breast cancer patients.** Ctrl: healthy controls; B = benign diagnoses; Tis = Tumor in situ; S = Stage.

Ovarian cancer

Female controls only were utilised in analysis of ovarian carcinoma group as in the case of breast cancer. Results assessed by both PicoGreen assay and qPCR with LINE-97 showed that levels of cfDNA were significantly higher in patients with stages I-III (n=9) compared to healthy cases (n = 10) ($p = 0.002$ for PicoGreen and $p = 0.001$ for qPCR). cfDNA concentration seems to correlate with increasing stage of the disease, resulting in highest values being obtained from patients with stage III ovarian carcinoma (fig.21) and correlation coefficient being $R^2 = 0.601$ for qPCR and $R^2 = 0.593$ for PicoGreen ($p = 0.01$).

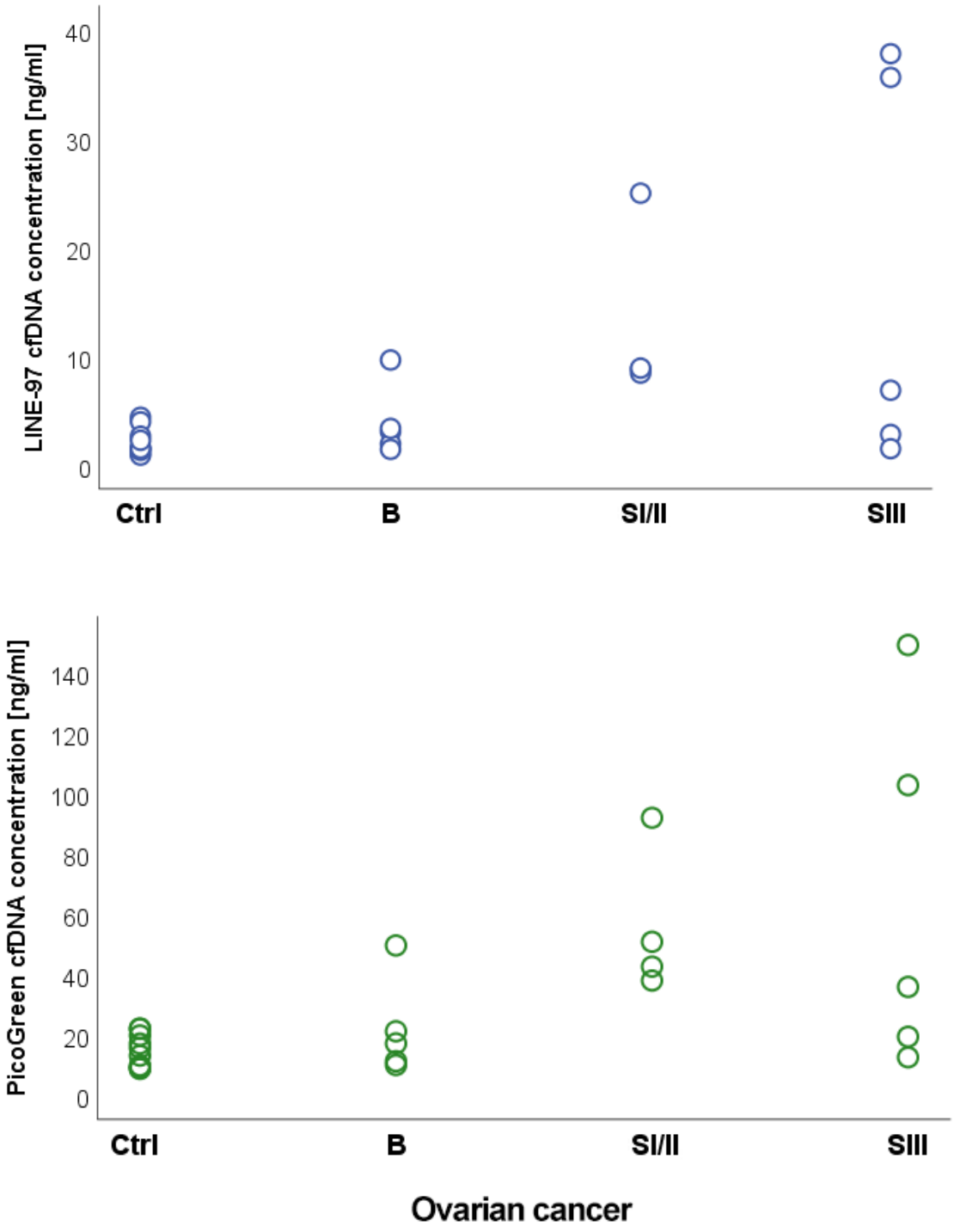

Figure 22. Scatter plot of cfDNA concentration measured by qPCR by LINE-97 and PicoGreen assay in ovarian cancer group. Ctrl = healthy controls; B = benign diagnoses; S = Stage.

Colorectal cancer

In this group, significantly elevated cfDNA levels in contrast with controls were statistically significant only using qPCR (p = 0.02). On the other hand, results from PicoGreen assay have not indicated significant difference between cancer and healthy individuals (p = 0.696) (fig.23, tab.15).

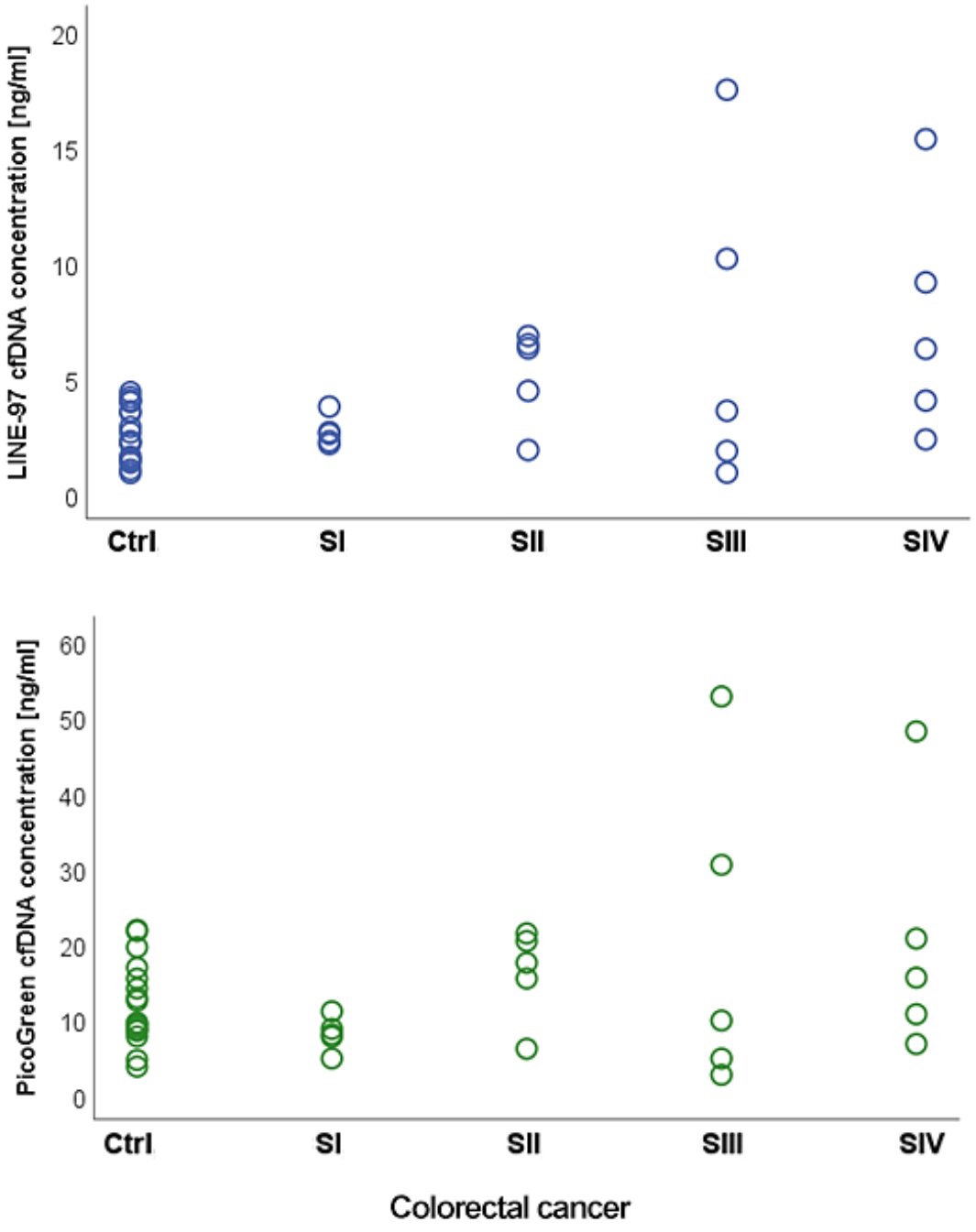

Figure 23.CfDNA concentration measured by qPCR by LINE-97 and PicoGreen assay in colorectal cancer group. Ctrl = healthy controls; S = Stage.

Pancreatic cancer

Similarly as in colorectal cancer group, significantly higher cfDNA concentrations in comparison with healthy controls were observed only using PicoGreen assay ($p = 0.01$) (fig.24). Concentration of cfDNA in stage IV samples was one of the highest of all samples (108.7 ± 106.8 ng/ml for PicoGreen and 21.0 ± 18.5 ng/ml for LINE-97 qPCR). qPCR results have shown an increase in concentration near to statistical significance ($p = 0.057$).

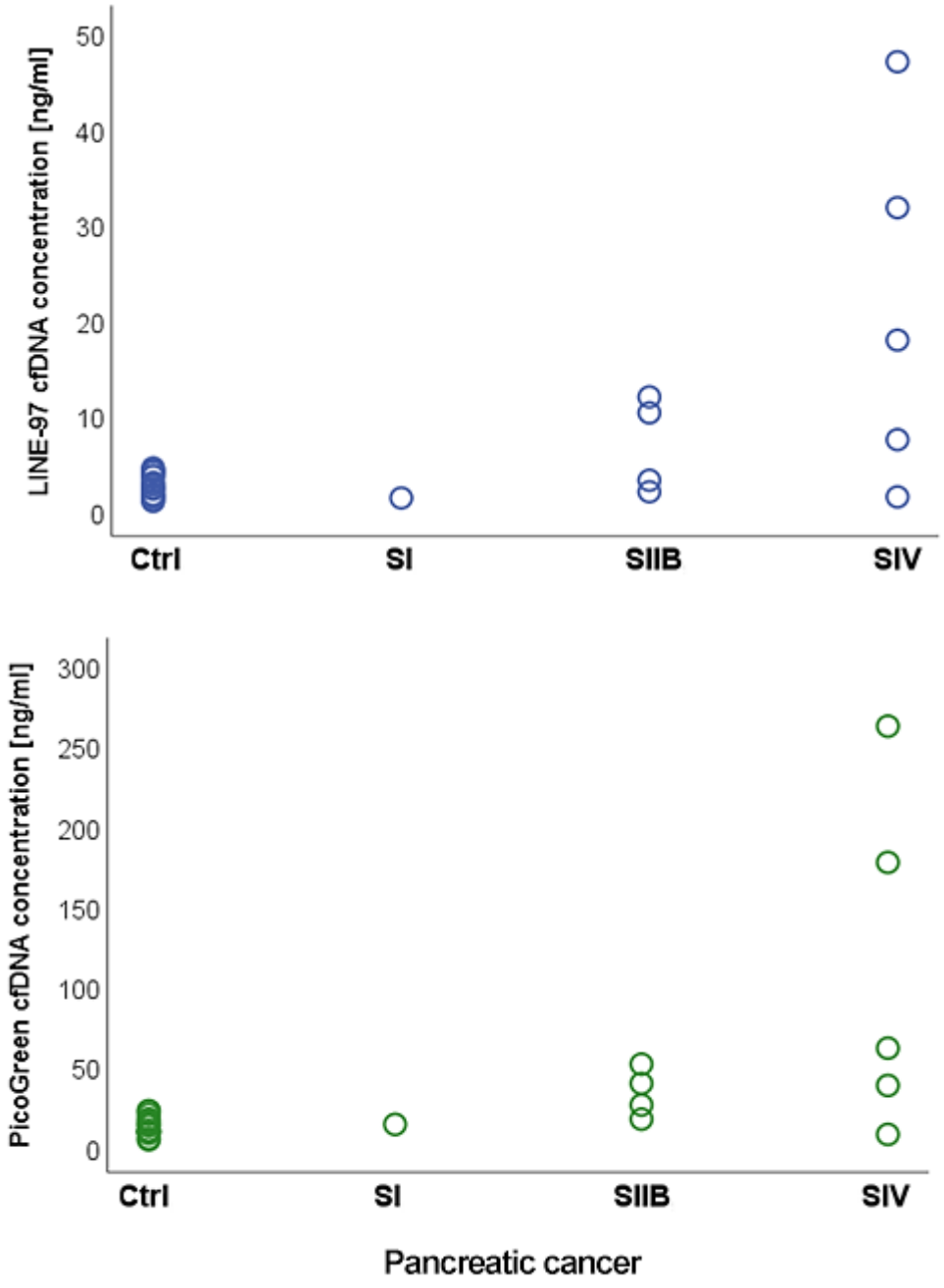

Figure 24.CfDNA concentration measured by qPCR using LINE-97 and PicoGreen assay in pancreatic cancer group. Ctrl = healthy controls; S = Stage.

During further analysis of additional information, the Spearman correlation test has not revealed any significant correlation between the age of patients (n = 69) and cfDNA concentrations in their plasma samples (tab.14).

	Mean	SD	Correlation coefficient	p-value
Age (n=69)	62.0	11.9		
qPCR cfDNA conc.	11.0	41.9	0.06	0.604
PicoGreen conc.	11.9	44.8	0.03	0.836

Table 14. Mean values measured using qPCR and PicoGreen assay correlated with age of patient.

Group and method	compared subgroups	n	mean	SD	p value
Breast carcinoma					
PicoGreen	controls Stage I-III	10 15	14.3 48.5	5.4 32.9	$p < 0.001$
qPCR LINE-97	controls Stage I-III	10 15	2.1 13.5	1.2 12.1	$p < 0.001$
Ovarian carcinoma					
PicoGreen	controls Stage I-III	10 9	14.3 60.0	5.4 44.9	$p = 0.002$
qPCR LINE-97	controls Stage I-III	10 9	2.0 15.1	1.2 13.9	$p = 0.001$
Colorectal carcinoma					
PicoGreen	controls Stage I-IV	18 20	11.8 16.1	5.4 13.7	NS
qPCR LINE-97	controls Stage I-IV	18 20	2.6 14.4	1.2 13.3	$p = 0.02$
Pancreatic carcinoma					
PicoGreen	controls Stage I-SIV	18 10	11.8 68.9	5.4 83.4	$p = 0.001$
qPCR LINE-97	controls Stage I-SIV	18 10	2.6 13.3	1.2 15.1	NS

Table 15. Summary of results in all patient groups.

5.2.2.1. Concentration evaluated using ddPCR

Measurement was performed on 44 samples (fig. 25). Threshold for fluorescence intensity was set to 4000, which means that all droplets occurring above the threshold were evaluated as positive (fig.26). Each of the positive droplets then corresponded to one copy of the RNase P gene (Earl et al., 2015). The number of positive and negative droplets is stated in figure 25.

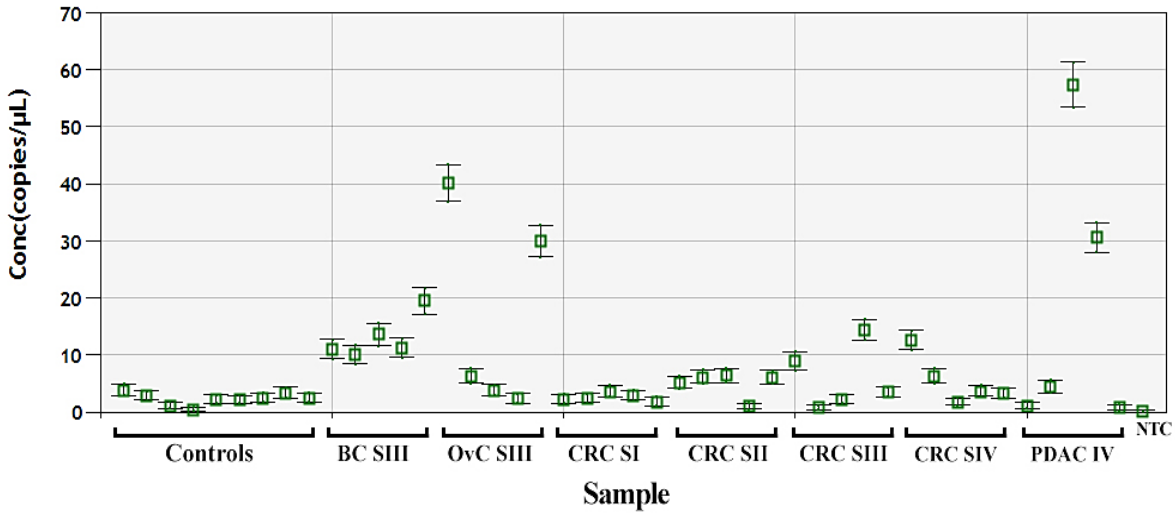

Figure 25. Graph of cfDNA concentration (copies/µl of reaction) measured using ddPCR,
NTC = non-template control.

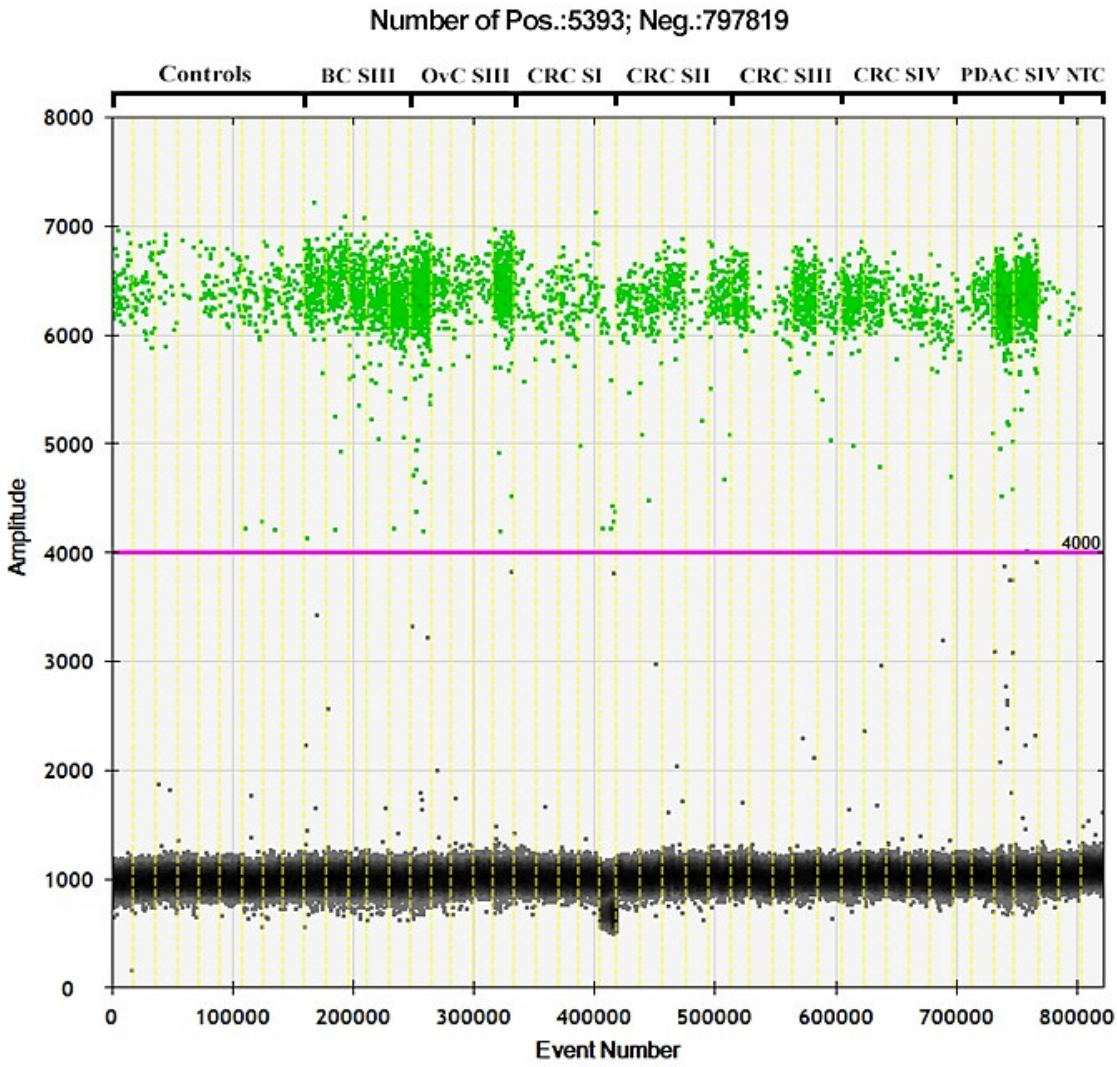

Figure 26. Number of positive (green) and negative (black) events. 5393 of positive and 797819 of negative droplets were observed.

Since all samples of colorectal cancer group were subjected to measurement, difference between individual stages and healthy controls was tested using the Kruskal-Wallis test. However, no significant difference was found (p = 0.255) (fig.27). Additionally, no significant changes were found in cancer patients compared to healthy individuals (p = 0.085). Samples of breast, ovarian and pancreatic cancer were measured in order to compare the method with values acquired using qPCR and PicoGreen assay.

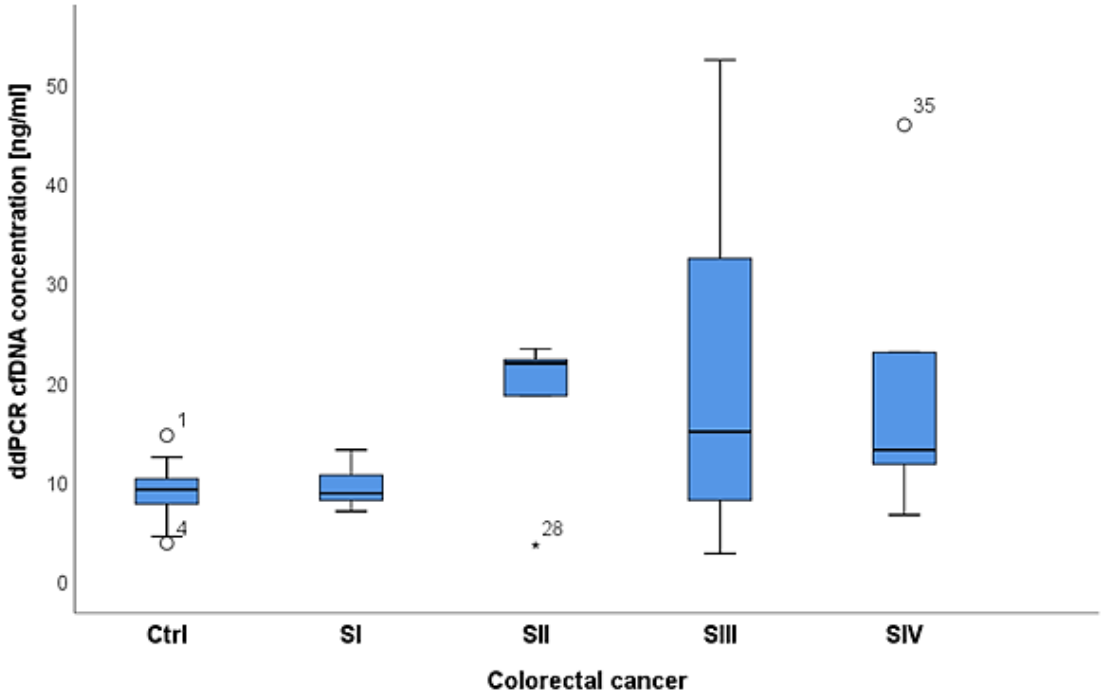

Figure 27. Boxplots with whisker-plots of cfDNA concentrations of individual stages in the colorectal cancer group compared to healthy controls. Boxes represent values within 25 – 75 percentiles with error bars and median. (○) = outliers; (*) = extreme outliers

The absolute numbers of the concentrations were calculated as amount of DNA per 1 ml of plasma sample, just as in case of PicoGreen and LINE-97 assays. The nonparametric Spearman test revealed strong correlation of the ddPCR with both qPCR LINE-97 ($p < 0.01$, $R^2 = 0.881$) and PicoGreen assay ($p < 0.01$, $R^2 = 0.867$) (fig.28).

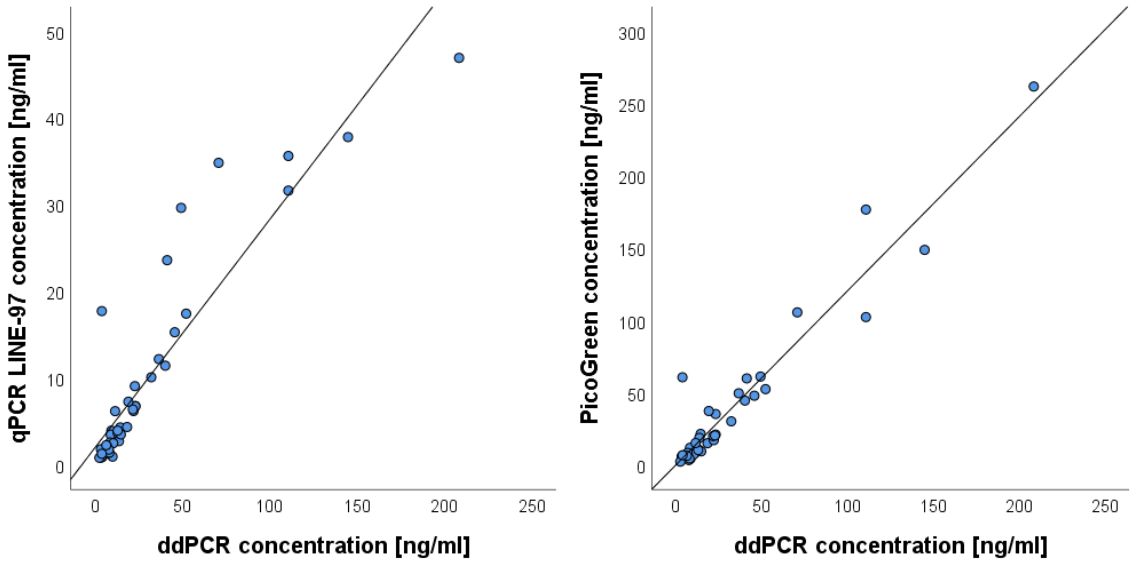

Figure 28. Linear regression curve for concentrations obtained by measurement methods.

a) ddPCR and qPCR assay, b) ddPCR and PicoGreen assay.

5.2.3. CfDNA integrity evaluation

CfDI was calculated as a ratio of LINE-266 to LINE-97 amplicons representing longer and shorter fragments of the cfDNA. Again, only female controls were used in cases of breast and ovarian cancer. Interestingly, cfDI of all patients (n = 69) negatively correlated with age (R^2 = -0.271; p = 0.024). Individual groups and stages of cancers are distinguished by different colour markers (fig.29).

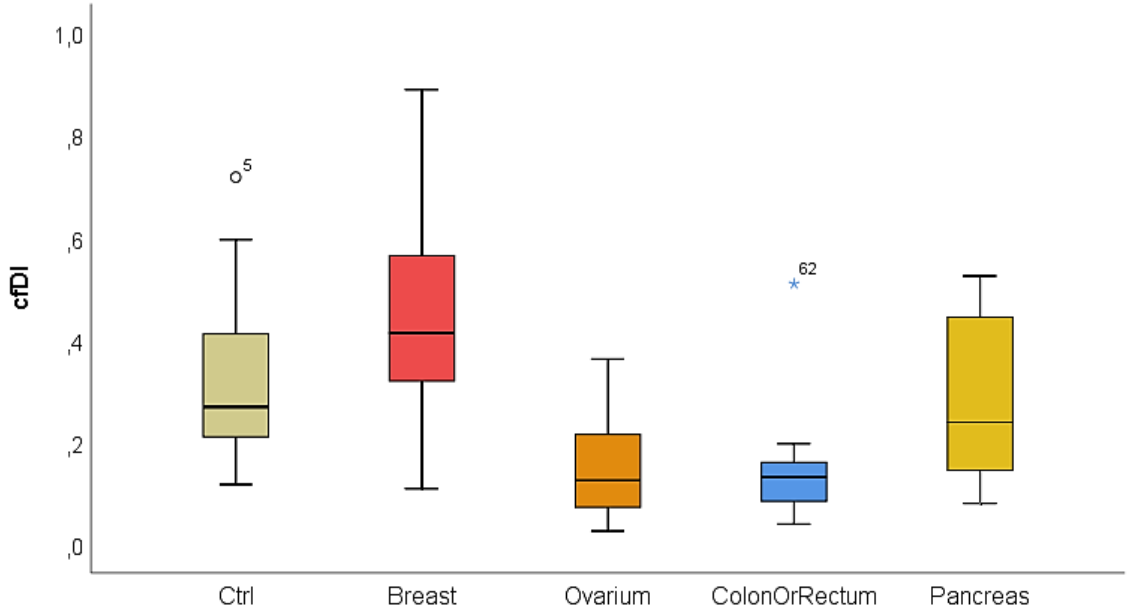

Figure 29. Boxplots with whisker-plots of individual groups' cfDI. Boxes represent values within 25 – 75 percentiles with error bars and median. (○) = outliers; (*) = extreme outliers.

Surprisingly, no significance was observed in breast cancer group when compared to healthy controls and benign cases (p = 0.093) (fig.30). On the other hand, cfDI was significantly lower in ovarian cancer patients than in controls (p = 0.001). Furthermore, the level of cfDI was decreased in higher disease stages (fig.31). The Spearman correlation test showed significant negative correlation of cfDI with stage of the disease (R^2 = -0.644) (p = 0.001).

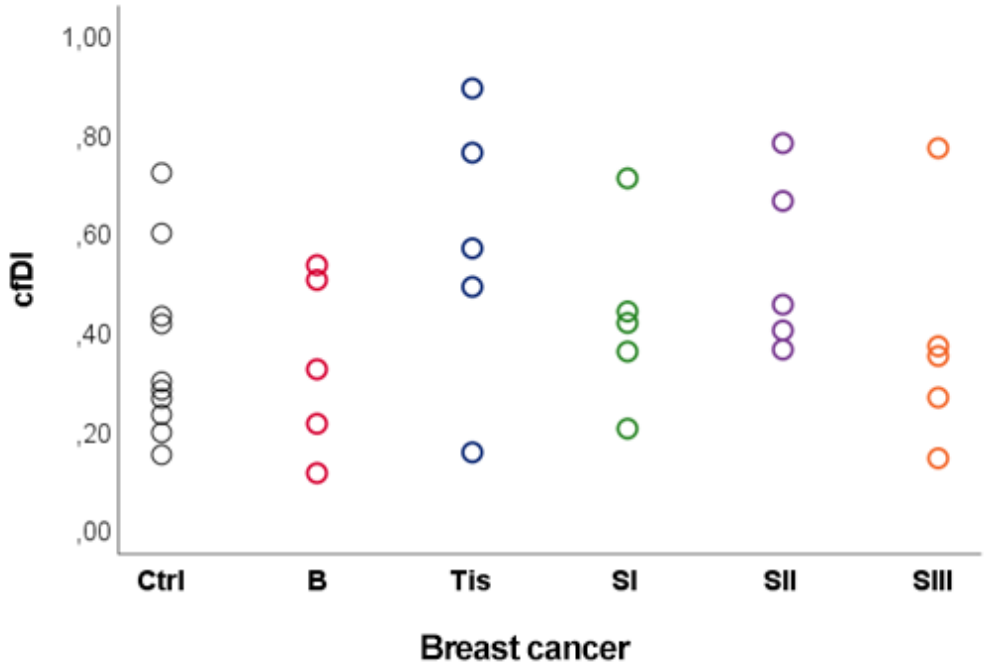

Figure 30. Scatter plot of cfDI in breast carcinoma patients. Ctrl = controls; B = benign diagnoses; Tis = tumour in situ; S = Stage.

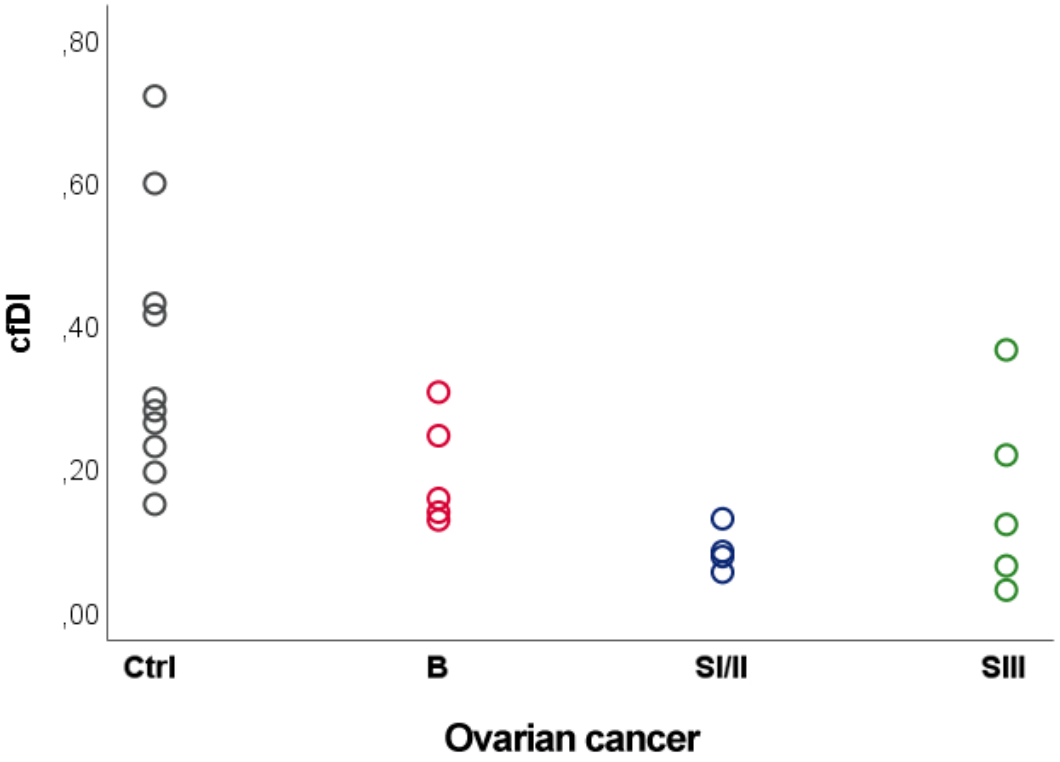

Figure 31. Scatter plot of cfDI in ovarian carcinoma group.

Ctrl = controls; B = benign diagnoses; S = Stage.

In the group of colorectal cancer, cfDI was also significantly lower in patient samples (n = 20) compared to the healthy controls (n = 18) ($p < 0.001$) (fig.32), similarly to the ovarian cancer group. Negative correlation of cfDI with stage of the disease was observed as well, where the healthy controls had the highest cfDI values ($R^2 = - 0.718$, $p < 0.01$).

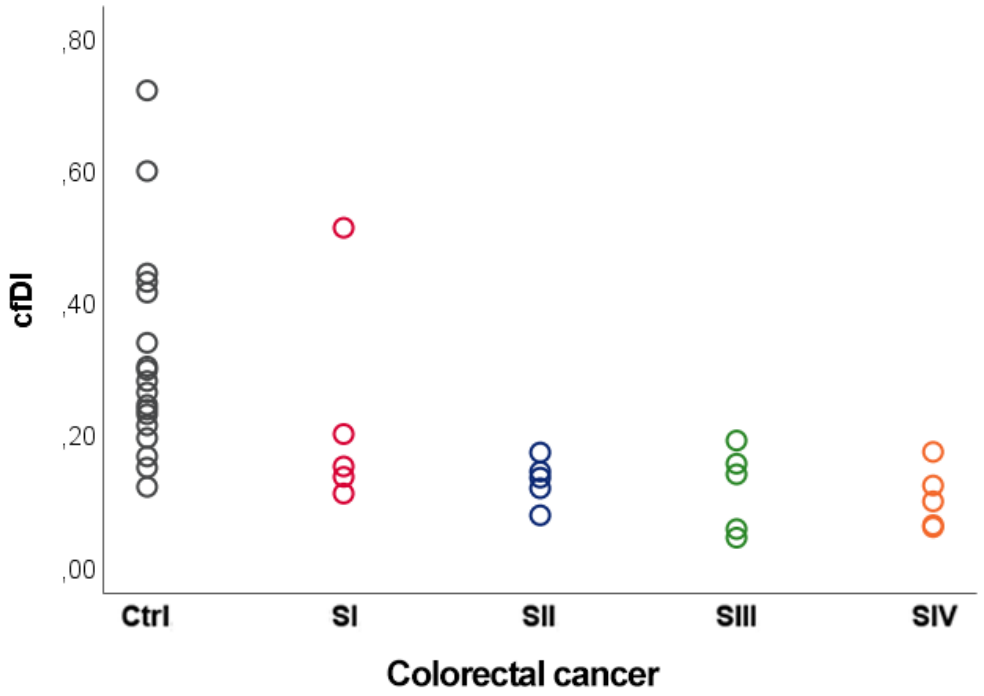

Figure 32. Scatter plot of cfDI in colorectal carcinoma group. Ctrl = controls; S = Stage.

CfDI of the pancreatic cancer group did not significantly differ from healthy individuals (p = 0.654) (fig.33). Summary of samples from all groups along with the p-values are presented in the table 16.

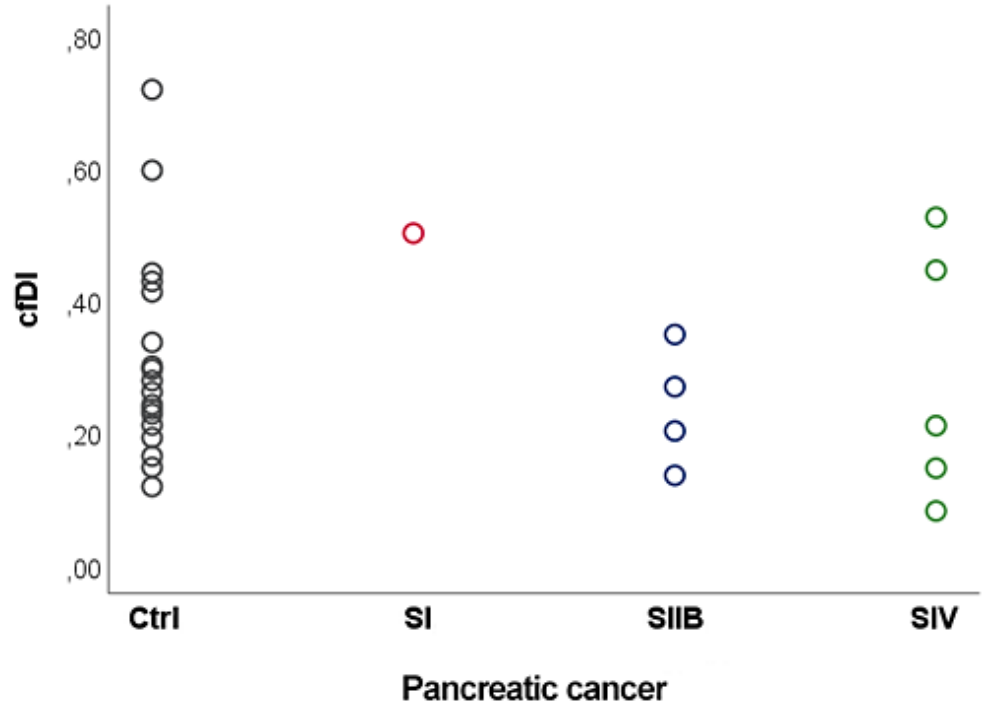

Figure 33. Scatter plot of cfDI in pancreatic carcinoma group. Ctrl = controls; S = Stage.

Group and method	compared subgroups	n	mean	SD	p value
Breast carcinoma					
cfDI	controls	10	0.4	0.2	NS
	Stage I-III	25	0.5	0.2	
Ovarian carcinoma					
cfDI	controls	10	0.4	0.2	p = 0.001
	Stage I-III	9	0.1	0.1	
Colorectal carcinoma					
cfDI	controls	18	0.3	0.2	p < 0.001
	Stage I-IV	20	0.1	0.1	
Pancreatic carcinoma					
cfDI	controls	18	0.3	0.2	NS
	Stage I-IV	10	0.3	0.2	

Table 16. Summary of cfDI in compared groups.

NS = non-significant results.

6 Discussion

CfDNA is a subject of research associated with pregnancy, neurological and inflammatory diseases, but particularly with cancer biology (Frank 2016; Everett and Chitty, 2015). Approximate concentration of cfDNA in plasma of healthy individuals ranges around 13 ng/ml, whereas in cancer patients might occur in levels as high as 100 ng/ml (Jahr et al., 2001; Oliveira and Hirata, 2018). However, low concentrations of cfDNA in blood present the greatest obstacle when it comes to the reliable analysis of its even smaller ctDNA fraction in cancer patients. The fact that detecting ctDNA might be quite challenging can be observed from heterogeneous results done by independent laboratories (Malentacchi et al., 2015). Low cfDNA levels are difficult to detect by conventional molecular methods and are easily lost during improperly validated isolation and measurement procedures. Furthermore, they are prone to possible contamination with other low-molecular-weight DNA molecules. Optimisation and standardizing of protocols is, therefore, an important part of overall cfDNA analysis process.

6.1. cfDNA isolation

The differences in results between many publications, such as varying cfDNA concentrations, might be explained by utilisation of various kits from different commercial sources. In this study, two kits Norgen serum/plasma purification mini kit and QIAamp CNA kit were tested on four plasma and sera samples. Even though the principle of both isolation kits is similar, higher yields on the average were obtained with QIAmp CNA kit as it seems that carrier RNA included in this kit aids higher recovery of cfDNA. This is in concordance with other studies that compared cfDNA isolation kits (Diefenbach et al. 2018; Solassol et al., 2018; Sorber et al., 2017; Warton et al. 2018). Based on the obtained results and literature, it was decided to utilise QIAmp CNA kit for subsequent cfDNA analysis, as it is one of the most commonly used.

The yields of cfDNA from serum differed from plasma samples quite fairly. As already described in the chapter *Limitations* and according to other studies, concentrations of cfDNA in serum are much higher, which is also apparent from the results of this study. The question remains, whether the serum contains a higher level of background DNA from leukocytes or even the apparent cfDNA itself can origin from lysed leukocytes due to the clotting process (Lee et al., 2001). Despite higher serum cfDNA concentrations,

KRAS allelic frequencies found in ctDNA were observed in smaller amounts in serum compared to plasma samples (Kloten et al., 2017). For these reasons, plasma samples were utilised in the following the analysis. However, a number of studies still use the serum as a source of cfDNA, which could affect the general accuracy of cfDNA as a biomarker. Apparently, utilisation of plasma or serum seems to be a matter of individual opinion, and both are widely used for cfDNA and cfDI quantification.

6.2. Optimisation of quantification methods

PicoGreen assay is frequently used sensitive spectrofluorometric method employing PicoGreen stain which binds preferentially dsDNA. Serial dilution of a standard lambda DNA from 0.2 to 100 ng/ml to "low-range" protocol has brought a remarkable change in cfDNA yields, as seen on sample P464 (tab.12*, 5 Results, 5.1.2. Quantification Methods*).

On the other hand, the optimisation of qPCR assays was accompanied by many obstacles. The main issue was persisting detection of non-specificities. First set of primers targeting Alu-repetitive DNA elements, ALU-115 and ALU-247, amplified quite a large amount of non-specific products in nearly all reactions. Alternative annealing temperatures and different primer concentrations did not resolve this problem as non-specific products were still detectable. Another tested ALU-111/260 primer pair behaved similarly as heterogeneous inconsistent peaks were observed in melt curve analysis. According to Funakoshi et al., a potential chance that ALU primers are targeting different sites within Alu repetitions due to their sequence similarity provides a possible explanation for the observed low specificity of Alu-based assays (Funakoshi et al., 2017). Because of repeated unsatisfactory results obtained by this assay, ALU primers were no longer utilised for the optimisation.

Another primer pair detecting ß-actin gene was previously reported to effectively quantify cfDNA (Szpechcinski et al., 2016). The *ACTB* primers seemingly performed very well as the R^2 value and efficiency of the reactions were optimal and no signal was detected in the NTC. However, the double peak of the melt-curve in ACTB-382 reactions implicated non-specificities, regardless of annealing temperature or employed master mixes of 4 different companies. Separation of the products from these qPCR reactions on electrophoretic gel did not reveal presence of primer-dimers (data not shown). No additional target sequences of ACTB-382 primers were found while checking their specificity in the BLAST database (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The most possible explanation for the double peaks in melt curve is contamination.

In contrast, LINE-based assay seemed to perform the best. Both R^2 value and efficiency were satisfactory, along with one single peak of each sample in melt curve analysis implying the absence of non-specificity in the reactions. Nevertheless, a signal remained in the NTC despite repeated utilisation of UNG in LINE-97 reactions under different conditions. Both ALU and LINE1 primers amplify the DNA fragments even in samples with very low concentration of cfDNA. This is thanks to highly abundant repetitive DNA elements in human genome that serve as annealing sites of the primers (Madhavan et al., 2014). This feature may be the reason why even a little amount of contaminating DNA can cause positivity of the NTC. Non-specificities occurring in LINE assay were previously reported by other authors (Madic et al., 2012; Rago et al., 2007) explained by unavoidable human DNA contamination present on equipment and chemicals (Urban et al., 2000, cited from Rago et al., 2007). However, careful utilisation of brand new chemicals and kits from different companies has not helped to solve this obstacle. The origin of NTC products could not be examined during this study. To confirm that these products may be non-specific amplicons of similar length, further analysis such as sequencing is needed. We acknowledge that the persisting positive NTC is indeed a limitation of this assay. However, the signal from the NTC was very weak and the concentrations were below an "effective laboratory background", which was set to 0.0174 pg/µl (Urban et al., 2000, cited from Rago et al., 2007). Therefore, the detection limit of this assay allows measuring of cfDNA despite of the signal in late Ct cycles of the NTC.

6.3. Evaluation of cfDNA concentration in cancer patients

PicoGreen and qPCR assays were optimised to evaluate the cfDNA concentration as accurately as possible. To recapitulate, the concentration was assessed by qPCR as the amount of all cfDNA fragments amplified by LINE-97 primers and by spectrofluorometric PicoGreen assay adjusted to low concentrations. Many authors have reported increased concentrations of cfDNA in the blood of patients with various types of cancer, including breast, ovarian, colorectal and pancreatic cancer (Giacona et al., 1998; Umetani et al., 2006; Zaher et al., 2013; Zhang et al., 2018). Results obtained in this study are in concordance with these findings, as significantly elevated levels of cfDNA in patients with mentioned diseases were demonstrated. Nevertheless, decreasing concentrations of cfDNA with the progression of prostate cancer using Alu-based assay were also reported (Arko-Boham et al., 2019), suggesting that reliability of these assays needs to be further investigated and all process of cfDNA assessment validated for each type of cancer.

CfDNA concentrations in the breast cancer group were observed to be increased compared to healthy subjects. A slight trend of rising cfDNA levels with an increasing stage is noticeable from figure 20 (*5 Results, 5.2.2. Quantification of cfDNA*). However, cfDNA levels of benign cases resemble those of carcinomas', which suggests low accuracy of cfDNA as a stage-specific marker in breast cancer. Similarly, in the case of ovarian cancer, no statistically significant difference was found between benign cases and healthy controls ($p = 0.371$ for PicoGreen assay and $p = 0.254$ for qPCR). Findings like these where no significant difference between benign and healthy cases was observed have been already reported by other authors as well (Shao et al., 2015). Benign cases and their cfDNA levels require further research in an additional study, as some of them might be precursors of the emerging disease. However, distinguishing benign cases from cancer patients or healthy individuals based on cfDNA still represents a challenge (Zaher et al., 2013).

In the colorectal carcinoma group, significantly elevated cfDNA levels of patients compared to controls were obtained by qPCR method only ($p=0.02$). CfDNA levels evaluated by PicoGreen assay had a very similar tendency compared to those obtained by qPCR, however, not statistically significant ($p=0.696$). It seems that in this case, high degree of fragmentation might provide a potential explanation for the possible lower specificity of PicoGreen assay, which will be further discussed in *6.4 Evaluation of cfDI index*.

Finally, cfDNA concentration of samples in stage IV of pancreatic cancer group was highest out of all patients (mean ± SD 108.7 ± 106.8 ng/ml for PicoGreen and 20.0 ± 18.5 ng/ml for LINE-97 qPCR). This time, significantly increased concentration in patients compared to controls was evaluated only using PicoGreen ($p=0.001$), although those using LINE-97 qPCR were near significance ($p=0.057$). Results of our measurements are consistent with other studies, in which mostly Alu-based qPCR assays were used to demonstrate elevated levels of cfDNA (Hao et al. 2014; Sikora et al. 2015; Umetani et al. 2006; R. Zhang et al. 2018). LINE-1 based qPCR was used only in a few publications (Madhavan et al. 2014; Cheng et al., 2017), but correlated with results obtained by Alu-assay.

Based on the observed results of this study, PicoGreen and qPCR assay strongly correlated. Interestingly, cfDNA concentrations obtained by the qPCR assay were nearly 4 times lower on average compared to concentrations obtained by PicoGreen. Similarly, other authors such as Szpechcinski et al. demonstrated several-fold higher concentrations of plasma DNA measured by PicoGreen compared to qPCR assay in NSCLC patients. The research group suggested an explanation that PicoGreen assay detects all the DNA fragments in the

sample, whereas SYBR Green of qPCR assay only the amplifiable DNA (Szpechcinski et al., 2008).

Furthermore, the results of the absolute measurement of cfDNA concentrations by ddPCR revealed a strong correlation with both qPCR and PicoGreen assays. DdPCR is generally considered to be more sensitive and reliable than the two latter mentioned (Whale et al., 2017). However, the results preclude us making any conclusions, mainly due to two factors: firstly, the ddPCR assay was not optimized and thus not validated for purposes of this study; secondly, an only small number of samples could have been used for the measurement. Therefore, measurement of cfDNA samples with ddPCR requires further research. Dynamics of cfDNA concentrations was already demonstrated to be potential prognostic biomarker utilizing both qPCR and ddPCR (Diehl et al. 2008).

6.4. Evaluation of cfDI index in cancer patients

The most interesting results were probably obtained by evaluation of the cfDI index. In recent years, cfDI was studied for its potential as a biomarker. A meta-analysis by Wang et al. even reported that Alu-based cfDI was more accurate diagnostic biomarker in detecting early stages of colorectal cancer than concentrations of cfDNA (Wang et al. 2018). A number of publications focused on cfDI is rising, although along with contradictory results. Umetani et al., the group which developed short and long-fragment Alu-based qPCR assay, demonstrated significantly increased serum cfDI in breast patients which correlated with tumour size (Umetani et al. 2006). Over time, most of recent studies reported significantly increased level of cfDI using mostly Alu or *ACTB*-based qPCR assay, for example in serum or plasma of breast (Iqbal et al., 2015; Kamel et al., 2016), ovarian (Zhang et al., 2018) or colorectal cancer patients (Hao et al., 2014).

In contrast, our results have shown a different trend. Although cfDI in breast and pancreatic cancer samples did not differ from healthy controls, significantly lower cfDI in plasma of colorectal and ovarian cancer patients were observed. Interestingly, cfDI even negatively correlated with the stages of studied diseases, which suggests that portion of shorter fragments dominates in higher stages. These findings are similar to results obtained in study by Madhavan et al., from which was adapted the qPCR assay protocol in the present study. They estimated cfDI from the plasma of four groups, where the highest value of cfDI was detected in healthy individuals, lower in primary breast cancer patients, followed by CTC-negative metastatic breast cancer patients and lowest values were observed in CTC-positive metastatic breast cancer patients (Madhavan et al. 2014). Another study carried out

by Yoruker et al. has reported similar results, i.e. decreased cfDI in colorectal patients' serum compared to healthy controls (Yörüker et al., 2015). Additionally, Cheng et al., who used qPCR with primer pairs adapted from the study by Madhavan et al. as well, have found that cfDI index was able to distinguish recurrent from non-recurrent breast cancer, as cfDI was significantly decreased in recurrent patients compared to non-recurrent (Cheng et al., 2018).

These observations support a hypothesis, that tumour-derived DNA may be highly fragmented, as cfDI is calculated as a ratio of longer to both shorter and longer fragments present in the blood. The high degree of fragmentation of ctDNA was already reported by other studies. For example, Mouliere et al. detected highly fragmented cfDNA in plasma of patients with metastatic breast cancer by amplifying fragments of various length targeted on *KRAS* gene sequence (fig.34). Additionally, they observed a very similar cfDNA fragmentation pattern in xenograft mouse model (Mouliere et al. 2011). Moreover, another experiment by Mouliere et al. have shown that specific selection of fragments as short as 90 – 150 bp enriched mutant ctDNA fraction from 0.9 fold up to 11 fold and enabled to detect aberration such as CNV that were previously undetectable (Mouliere et al., 2018). Therefore, size distributed detection of ctDNA could potentially improve assays to solve problems with noisy background of wild-alleles.

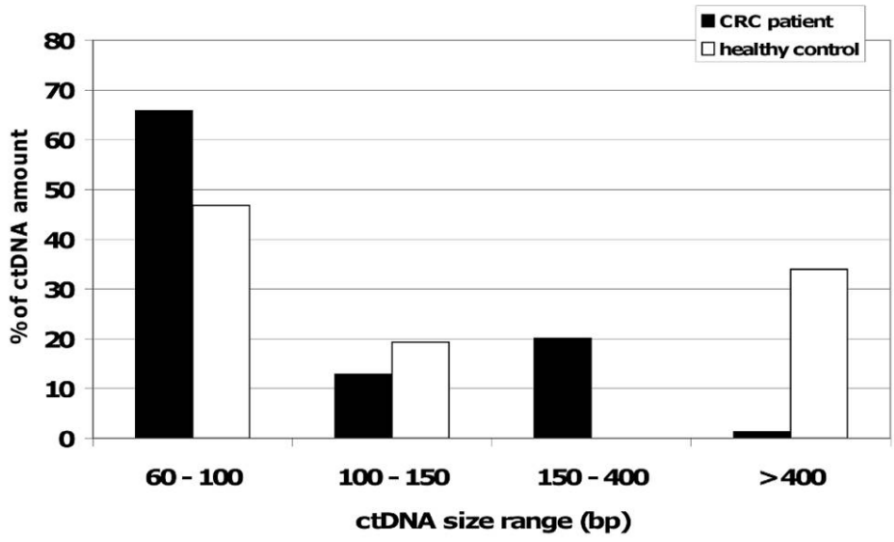

Figure 34. Amount of KRAS gene ctDNA amplicons categorized by size (Adapter from Mouliere et al. 2011).

Later on, a similar pattern was also observed by Jiang et al., who reported the presence of aberrant short and long fragments in plasma of hepatocellular carcinoma patients using massive parallel sequencing (Jiang et al. 2015).

The question remains how many factors affect the amount and length of cfDNA fragments. Therapy may be indeed one of these factors. Cheng et al. observed decreased cfDNA concentration and increased cfDI index after the first cycle of systemic therapy compared to baseline levels, using both Alu and LINE-assay (Cheng et al. 2018). On the other hand, there were other studies which have reported a decrease of cfDI after therapy (Agostini et al., 2011) or after surgery (Hao et al., 2014; Iqbal et al., 2015) using Alu-based assay in colorectal and breast cancer. Apparently, the utilisation of cfDI as a predictive biomarker requires further research.

Selection of an isolation method represents another factor playing an important role. Devonshire et al. compared four different isolation kits (including QIAamp CNA kit) and observed differing yields of cfDNA fragments varying in lengths, specifically lower yields of shorter than longer fragments, depending on used kit (Devonshire et al. 2014). Additionally, another study observed only di-nucleosomal form of cfDNA found in one of six plasma pools from 24 lung cancer patients, which was isolated by QIAamp CNA kit. Authors hypothesised that cfDI may be dependent on the particular isolation kit (Solassol et al. 2018). Based on these findings, the selection of an isolation method may also affect cfDNA fragment length. Thus, although QIAamp CNA kit is considered an effective gold standard for cfDNA isolation, it is necessary to admit that this approach may have unknown limitations which could potentially modify our results accordingly.

Moreover, preference of serum over plasma can affect also integrity of long fragments which may be released into the serum due to cell lysis during serum separation (Yu et al., 2014). Chan et al. isolated cfDNA from serum using an index of 201 bp and 105 bp amplicons and demonstrated significantly higher cfDI from serum in comparison with plasma, with even greater cfDI values using 356/105 bp ratio. Again, this suggests that a portion of cfDNA in serum may originate from hematopoietic cells (Chan et al., 2005).

The choice of particular primer pair is also an important aspect. Interestingly, there was a case when *ACTB*-based qPCR assay showed lower cfDI in colorectal cancer patients compared to healthy subjects, whereas Alu-based qPCR assay has shown only a small difference in these groups (Yörüker et al. 2015). Differences between utilised qPCR assays are proving that standardizing of protocols used for these purposes is essential.

Furthermore, Sedlackova et al. have conducted measurements by PicoGreen and Alu-based qPCR assays of differently fragmented DNA from blood of healthy individuals. Their results have shown that PicoGreen assay, as well as Alu-based qPCR assay, detected significantly lower concentrations of DNA when it was fragmented into small (150

bp) fractions (fig.35). This indicates that the measurement of cfDNA concentration may also be influenced by the degree of its fragmentation (Sedlackova et al., 2013). These limitations of PicoGreen assay could provide a possible explanation for our results in the colorectal cancer group. Patients in advanced stages had lower concentrations of cfDNA assessed by PicoGreen than by qPCR LINE assay. If we hypothesise that the highest fragmentation of cfDNA occurs in the advanced stages of cancer, it could explain that PicoGreen assay was affected by short fragments and measured the lower amount of cfDNA. Moreover, the advanced stages of this group had also low levels of cfDI, which could confirm the presence of a large portion of short cfDNA fragments (fig.31, chapter *5.2.3. CfDNA integrity evaluation*).

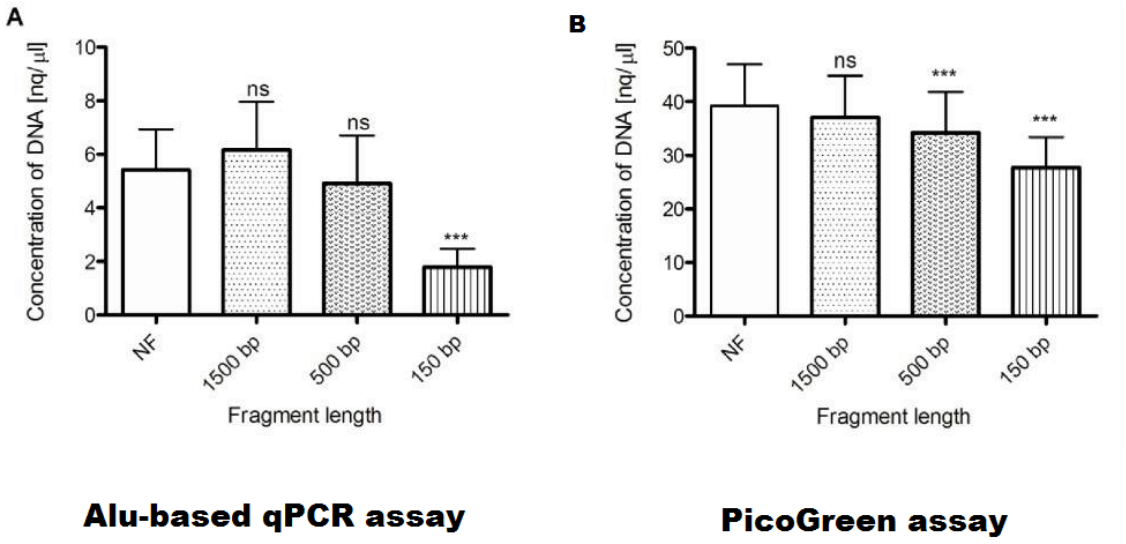

Figure 35. The DNA quantification of cfDNA using two different methods. (A) Alu-based qPCR assay with 10-fold diluted samples and (B) PicoGreen assay. DNA was fragmented into 1500bp, 500bp and 150bp fragments or non-fragmented (NF) (Adapted from Sedlackova et al. 2013).

Moreover, Alu-based qPCR assay is one of the most utilised approaches in a lot of studies to quantify the overall cfDNA concentration and cfDI. Sedlackova et al. presented the possibility of potential inaccurate measurements by Alu assay due to the presence of large proportion of short fragments (fig.34). Incorrect assessment of cfDNA concentration and its fragments could lead to affecting not only cfDI index but also general quantification of cfDNA concentration. It seems that cfDNA fragmentation plays an important role in terms of cfDNA quantification and cfDI evaluation. The facts listed above could possibly contribute to the clarification of heterogeneous results reported in the literature.

To summarise, cfDNA concentration and integrity assessment depend on many factors, such as the method of the extraction and quantification, but also the degree of cfDNA fragmentation. The greatest limitation of the present study is a small number of available samples. For example, in the case of pancreatic cancer group, no evident trend of cfDI levels among groups could be observed apparently due to the limited amount of samples. In fact, a lot of studies that focused on cfDNA and cfDI levels conducted their research on a similarly small number of patients. This field still requires improvements in standardising protocols with reproducible results and further analysis of the factors influencing the accuracy of cfDNA.

7 Conclusion

In many cases, the dynamics of cfDNA levels and presence of cancer-specific mutations showed better performance than conventional tumour markers or CTCs in respect to prognosis and prediction. The present study is divided into two parts: optimisation and validation of methods for effective isolation and quantification of cfDNA, and measurement of samples from cancer patients using previously optimized methods.

Firstly, our results provide validation of an effective isolation method selected from two commercial isolation kits. The QIAamp Circulating Nucleic Acid isolation kit (Qiagen) was chosen for cfDNA isolation and its further analyses. Moreover, the protocols of cfDNA quantification methods were optimized and validated as well. Points of the standard curves and compartments of PicoGreen and qPCR assay were adjusted to measure low concentrations of cfDNA.

In the second part, cfDNA concentration of cancer patients was estimated along with the qualitative analysis of cfDNA, the cfDI. The differences between cancer patients, their stages and healthy controls were evaluated. The results demonstrate increased cfDNA level in all examined cancer groups compared to controls. On the other hand, cfDI negatively correlated with a stage in ovarian and colorectal cancer groups and support the hypothesis that cfDNA derived from tumour may be more fragmented in comparison with DNA from healthy cells. These results prove that cfDNA is a promising candidate for more detailed study, as we acknowledge limitations of the methodology and the possibility of improvements.

Assessment of cfDNA parameters is generally accompanied by shortcomings, which remain the main limitation of cfDNA and its smaller fraction, ctDNA. Specificity and sensitivity of cfDNA are often insufficient or can be improved with expensive and experimental techniques as the next-generation sequencing. Although many studies are dedicated to the cfDNA detection approaches, the amount of heterogeneous data is rising together with the number of publications. Standardized blood processing, isolation kits, protocols and measurement methods are required for reliability and reproducibility of the results. Nevertheless, the impressive potential of cfDNA as a biomarker is very apparent and can surely provide a valuable tool in combination with other biomarkers for improvement in the accuracy of personalised medicine.

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9 Attachments

9.1. Attachment I

The presentation of results of this thesis on conference
XVIII. Setkání biochemiků a molekulárních biologů v Brně, 14.–15. 11. 2017.

Masarykova univerzita – Přírodovědecká fakulta,
Ústav biochemie a Národní centrum pro výzkum biomolekul
a
Česká společnost pro biochemii a molekulární biologii

pod záštitou

rektora Masarykovy univerzity doc. PhDr. Mikuláše Beka, Ph.D.
děkana Přírodovědecké fakulty MU doc. RNDr. Jaromíra Leichmanna, Dr.

XVIII. setkání biochemiků a molekulárních biologů

Sborník příspěvků

14. – 15. listopadu 2017

Konferenční centrum hotelu Continental v Brně

PŘEDEŠLÁ SETKÁNÍ BIOCHEMIKŮ A MOLEKULÁRNÍCH BIOLOGŮ V BRNĚ

14. července 1997; 21. ledna 1998; 3. února 1999; 9. února 2000;
14. února 2001; 7. února 2002; 29. ledna 2003, 3.–4. února 2004;
9.–10. února 2005; 8.–9. února 2006; 31. ledna–1. února 2007;
6.–7. února 2008; 14.–15. dubna 2009; 20.–21. dubna 2010; 1.- 2.11.2011;
speciální sekce na XXIII. Biochemickém sjezdu 26. - 29. srpna 2012; 11.-12. listopadu 2014;
10.-11. listopadu 2015
http://orion.chemi.muni.cz/Setkani/index.htm

ANALÝZA CIRKULUJÍCÍCH MARKERŮ U PACIENTŮ SE SOLIDNÍMI NÁDORY

Katarína Buranovská [1,2], Veronika Brynychová [2,3], Radka Václavíková [2,3], Renata Koževníkovová [4], David Vrána [5], Jiří Bouda [6], Pavel Souček [2,3]

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Cirkulujúca DNA (cell free DNA, cfDNA) je považovaná za inovatívny prognostický a prediktívny biomarker vo svete onkologickej diagnostiky. V mnohých prípadoch onkologických pacientov bola pozorovaná odlišná koncentrácia a integrita cfDNA v porovnaní so zdravými jedincami, vrátane závislosti na štádiu ochorenia, čo sa osvedčilo ako efektívny marker pre včasnú diagnostiku a monitorovanie návratu ochorenia. Práca sa venuje problematike výhod použitia cfDNA u pacientov s rôznymi nádorovými ochoreniami, vrátane spôsobov stanovenia koncentrácie a integrity.

cfDNA sme izolovali pomocou komerčných kitov Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit (Norgen) a QIAamp Circulating Nucleic Acid (Qiagen) zo vzoriek plazmy a séra od pacientok s karcinómom prsníka a porovnali ich efektivitu. Koncentrácia cfDNA bola meraná fluorometricky pomocou optimalizovanej PicoGreen assay vzhľadom na nízke koncentrácie cfDNA. Pre stanovenie integrity cfDNA bola optimalizovaná qPCR s využitím fragmentov DNA repetitívnych elementov o rôznej dĺžke (ALU, LINE). V prvej fáze sme použili 48 vzoriek plazmy od pacientok s karcinómom prsníka a ovárií v rôznych štádiách ochorenia a 10 vzoriek od zdravých dobrovoľníkov.

Ako najideálnejší spôsob izolácie cfDNA sme zhodnotili použitie kitu firmy Qiagen, ktorým sme docielili najväčšie výťažky cfDNA. Pre výpočet integrity sme použili qPCR s primermi LINE1 pre kratšie a dlhšie fragmenty cfDNA (97 a 266 bp). Následne naše výsledky ukázali významne zvýšenú koncentráciu cfDNA v plazme pacientok s nádormi prsníka a ovárií v porovnaní so zdravými jedincami. Naopak integrita cfDNA bola u pacientok s karcinómom ovárií významne nižšia v porovnaní s kontrolami, u pacientok s karcinómom prsníka sa integrita cfDNA od kontrol nelíšila. Integrita cfDNA taktiež negatívne korelovala so štádiom ochorenia karcinómu ovárií.

So štúdiou naďalej pokračujeme so vzorkami plazmy od pacientov s nádorom pankreasu a hrubého čreva.

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Přírodovědecká fakulta, Masarykova univerzita v Brně

DIPLOM

nejlepší posterová prezentace

XVIII. setkání biochemiků a molekulárních biologů

Bc. Katarína Buranovská
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V Brně dne 15. listopadu 2017

9.2. Attachment II

The results of this thesis were presented on seminar meeting at Institute for Cancer Research, and University of Oslo (uiO) on Translational Cancer Medicine in the period from 6th to 9th June 2018 during exchange visit of student of Charles University and University of Oslo.

UiO : University of Oslo
Institute of Clinical Medicine

Date: 12 June 2018

Dear Sirs,

It was my pleasure to host Assoc. Prof. Pavel Soucek and his group and arrange a seminar for his and our PhD and MSc students at our Institute for Cancer Research, and University of Oslo (uiO) on Translational Cancer Medicine in the period from 6th to 9th June 2018.

Thereby, I would like to certify that the following students from Pavel Soucek´s group have been hosted at Oslo University and had talks with titles:

Maria Kovacova: Decision tree for prioritizing variants in oncogenes and pharmacogenes using combination of web-based and command-line tools in exome sequencing studies

Petr Holy: Variability in oxysterol-related genes in cancer

Karolina Seborova: Long Non-Coding RNA: significance in ovarian cancer

Kamila Koucka: NOTCH signalling pathway and its role and importance in patients with ovarian carcinoma

Katarina Buranovska: Analysis of circulating markers in patients with solid tumors

We all really enjoyed this seminar and stay of Czech colleagues here.

Many thanks for assisting in the organisation of this remarkable event,

Best wishes,

Vessela Kristensen
professor, PhD

Department for Clinical Molecular Biology (EpiGen), Faculty of Medicine, University of Oslo;
and Group of Cancer Genome Variation, Department of Genetics, Institute for Cancer Research, Oslo University Hospital-Radiumhospitalet, Oslo, Norway

All Nature is but Art, unknown to thee:
All chance, direction which thou canst not see;
All discord, harmony not understood.
Alexander Pope

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