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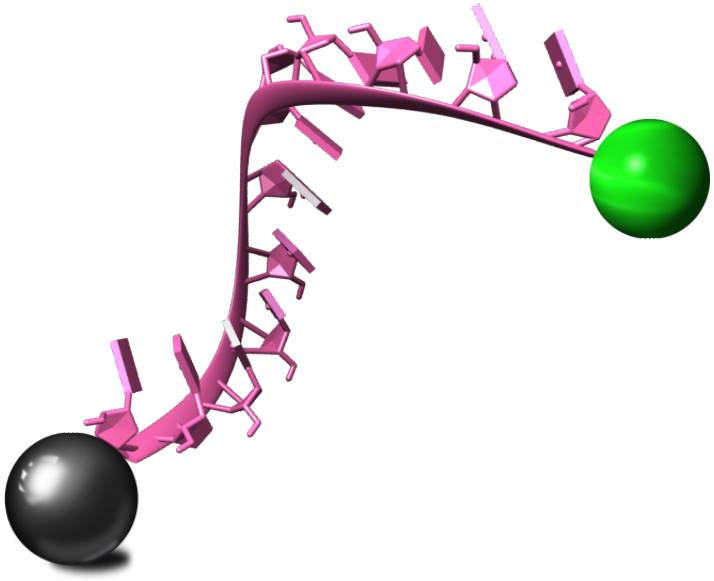
Alien Balian



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Nuclease Activity as a Biomarker in Cancer Detection

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During the course of the research underlying this thesis, Alien Balian was enrolled in Forum Scientium, a multidisciplinary doctoral program at Linköping University, Sweden.

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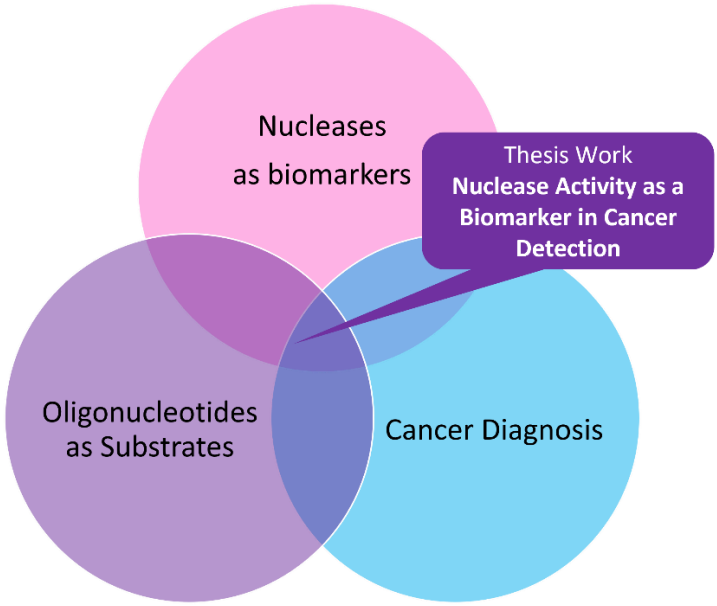
Cover image, a nucleic acid probe flanked with a fluorophore at one end and a quencher at the other end. The image is designed by Frank Hernandez and Alien Balian



To Ella, Amelinda and Rami

“The only thing greater than the power of the mind is the courage of the heart”
-John Nash

Abstract

Nucleases are a group of enzymes that cleave the phosphodiester bonds in nucleic acids. As such, nucleases act as biological scissors that exhibit a plethora of fundamental roles, in prokaryotes and eukaryotes, dependent or non-dependent on their catalytic capability. Thus, differential status of nucleases between healthy and disease conditions might not be surprising, and can be deployed in disease detection. Specifically, there is growing body of research demonstrating the potential of nucleases as diagnostic biomarkers in several types of cancer. Biomarkers for early diagnosis are an immense need in the diagnostic landscape of cancer. In this sense, nucleases are promising biomolecules, and they possess a unique feature of catalytic activity that could be deployed for diagnosis and future therapeutic strategies.
In this thesis we aim to demonstrate the use of nucleases as biomarkers associated to cancer, and the capability of oligonucleotide substrates for targeting a specific nuclease.

The thesis work begins with comprehensive review of nucleases as promising biomarkers in cancer diagnosis (paper I). Then, we provide a methodological study in paper II, in which we propose a flexible approach for detection of disease associated nuclease activity using oligonucleotides as substrates. The probes utilized here are flanked with fluorophore at the 5'-end and a quencher at the 3'-end. Upon cleavage by nucleases, the fluorescent signal is increased in a proportional fashion to nuclease activity. This platform is suitable to implement in detection of any disease in which nuclease activity is altered.
We have applied this method in paper III, by using 75 probes as substrates to screen breast cancer cells, along with controls, for nuclease activity. We have identified a probe (DNA PolyAT) that discriminates between BT-474 breast cancer cells and healthy cells based on nuclease activity profile associated with cell membrane. Next, we screened tissue samples from breast tumors for nuclease activity, and we have identified a set of probes with the capability to discriminate breast tumor and healthy tissues in 89% of the cases (paper IV). To achieve a step forward towards non-invasive diagnosis, we have developed an activatable magnetic resonance imaging (MRI)-probe (paper V). The MRI-probe is oligonucleotide-based that works like a contrast agent, and it is activated only in presence

of a specific nuclease. MRI-probes provide advantages over fluorescent probes, such as high spatial resolution and unlimited tissue penetration.
In conclusion, our findings suggest the utility of nuclease activity as a biomarker in cancer detection. Moreover, we demonstrate the applicability of nuclease activity-based approaches in imaging modalities, such as MRI. Our future aim is to translate our findings into non-invasive detection of breast cancer by utilizing breast cancer activatable MRI-probes.

Keywords: nucleases, nuclease activity, cancer detection, diagnosis, oligonucleotides, nucleic acid probes, MRI-probes, activatable probes.

Populärvetenskaplig sammanfattning

Nukleaser är en grupp enzymer som förekommer naturligt i kroppen. De har flera viktiga uppgifter på cell- och kroppsnivå. Till exempel spelar de stor roll i bevarande och redigering av arvsmassa, och i immunförsvaret mot patogener. Vidare fungerar nukleaser som biologiska saxar. De bryter ned nukleinsyrorna deoxiribonukleinsyra (DNA), och ribonukleinsyra (RNA), och heter därför DNaser respektive RNaser. En del nukleaser bryter dock ner båda sorters nukleinsyror utan någon substratpreferens. Mer specifikt katalyserar nukleaser hydrolys av fosfodiesterbindningar som länkar sockermolekylerna i den så kallade ”ryggraden” av nukleinsyrorna. Vidare har många studier bevisat att flera nukleaser är förändrade i sitt uttryck samt katalytisk aktivitet (nedbrytningsförmåga mot nukleinsyror) i samband med cancer. Vår hypotes är, baserat på detta, att vi skulle kunna upptäcka cancer genom cancerassocierade nukleaser, specifikt genom cancerassocierad nukleasaktivitet. I denna avhandling utnyttjas nukleasaktiviteten (katalytiska förmågan) som biomarkör för att detektera bröstcancer, genom att designa nukleinsyrasonder eller oligonukleotider (korta sekvenser av naturliga eller kemiskt modifierad DNA och RNA) som är specifikt klyvbara av cancer-nukleaser men inte av normala nukleaser. Syftet är att utveckla nya metoder för cancerdiagnostik baserat på cancer-nukleaser som målmolekyler och nukleinsyrasonder som upptäcksverktygen eller substrat till nukleaserna.

I den första uppsatsen i denna avhandling sammanfattar vi litteraturen rörande nukleaser som potentiella diagnostiska biomarkörer i cancer. Enligt litteraturen är flera nukleaser förändrade i proteinuttryck, genuttryck, och/eller katalytisk aktivitet i samband med flera former av cancer. Bröstcancer, prostatacancer, tarmcancer är några exempel. De flesta studierna undersöker diagonostiska potentialen på en nukleas genom att använda sedvanliga metoder för gen- eller proteinuttryckanalys. En markant mindre andel studier är genomförda på nukleasernas katalytiska aktivitet.

I den andra uppsatsen, beskriver metoden där vi designar oligonukleotider som är specifikt klyvbara av nukleaser förknippade med en sjukdom. Först designas en uppsättning oligonukleotider och används som substrat för att screena eller mäta nukleasaktivitet. Screeningen kan

göras i flera omgångar och i varje omgång skräddarsys oligonukleotiderna för att förbättra specificiteten till nukleaser förknippade med sjukdom. Den flexibla syntesen där kemiska modifieringar lätt kan integreras möjliggör en anpassad design av oligonukleotiderna. Oligonukleotiderna är kopplade med en fluorofor (lysande ämne) på det 5'-kolet och en släckare på det 3'-kolet. I början är oligonukleotiderna släckta på grund av närliggande fluoroforen och släckaren. När de bryts ner av nukleaser ökar avståndet mellan släckaren och fluoroforen som emitterar en signal som kan mätas och kvantifieras. På så sätt kan nukleasaktivitet mätas för den är proportionell till emitterade fluorescensintensiteten.

Vi tillämpar denna metod i den tredje uppsatsen där sjuttiofem oligonukleotider använts som substrat för att screena nukleasaktivitet på nio bröstcancercellinjer samt två kontrollcellinjer (friska celler). Vi har identifierat genom screeningen en oligonukleotid, DNA PolyAT, som kan särskilja mellan BT-474 bröstcancerceller och normalceller baserat på nukleasaktivitet associerad till cellmembranet. Denna oligonukleotid är nedbruten i dubbelt så stor grad av cancercellerna jämfört med kontrollen. I den fjärde uppsatsen har vi screenat vävnader från brösttumörer. Genom att kombinera screening med beräkningsanalys har vi identifierat tre oligonukleotider vilka tillsammans kan särskilja mellan cancertumörer och godartade tumörer. I den sista uppsatsen i denna avhandling har vi utvecklat en oligonukleotid-baserad aktiverbar magnetröntgen (MR)-sond. MR-sonder tillhandahåller visualisering med hög rumslig upplösning och obegränsad vävnadspenetration, vilket gör de mer kliniskt relevanta jämfört med fluorescensprober. MR-sonden i denna uppsatts kan upptäcka en specifik nukleasaktivitet och fungerar som MR-kontrastmedel.

Sammanfattningsvis tyder våra resultat på att cancerassocierad nukleasaktivitet är en potentiell biomarkör för cancerdiagnosticering, genom att använda nukleinsyrasonder som substrat eller molekylära detekteringsverktyg. Dessutom kan oligonukleotider implementeras i flera avbildningsmetoder t.ex. MR. Framtida mål är att kunna använda MR-oligonukleotider i icke-invasiv bröstcancerdiagnostik. Detta genom att utveckla MR-sonder baserade på oligonukleotiderna som särskiljer cancer och normala brösttumörer.

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Norrköping, December 2022

Alien Balian

Papers Included in the Thesis

I. Nucleases as Molecular Targets for Cancer Diagnosis
Balian A., Hernandez FJ.
Biomark Res., (2021), 9(1):86.

Author's contribution: AB has conducted literature search, written all the sections, made the initial design of the figures and responded to the reviewers' comments to edit the paper.

II. Kinetic Screening of Nuclease Activity Using Nucleic Acid Probes
Balian A., Garcia Gonzalez J., Bastida N., Akhtar K.T., Borsa B.A., Hernandez FJ.
Vis. Exp., (2019), (153).

Author's contribution: AB has written some of the sections, conducted experimental work, generated images and video captions and participated in replying to reviewer comments.

III. Exploring Nuclease Activity Profile of Breast Cancer Cells
Balian A., Hernandez LI., Hernandez FJ.
In Manuscript

Author's contribution: AB has planned and executed the experimental work, plotted, and discussed data, and written the manuscript.

IV. Discovery and Proof-of-Concept Study of Nuclease Activity as a Novel Biomarker for Breast Cancer Tumors
Hernandez LI., Araúzo-Bravo MJ., Gerovska D., Solaun RR., Machado I., **Balian A.**, Botero J., Jiménez T., Zuriarrain Bergara O., Larburu Gurruchaga L., et al.
Cancers, (2021), 13, 276.

Author's contribution: AB has contributed to writing some sections such as the introduction and abstract, conducted

confirmatory experiments using samples from Sweden, and participated in editing the paper and responding to the reviewers.

V. Activatable MRI Probes for the Specific Detection of Bacteria
Periyathambi P, **Balian A.**, Hu Z, Padro D, Hernandez, LI., Uvdal K, Duarte J, Hernandez FJ.
Anal Bioanal Chem., (2021), 413(30)

Author's contribution: AB has conducted a part of the experimental work, mainly by replicating the results of coupling and performing toxicity assays. AB has written the cell culture and cytotoxicity assay sections and participated in editing the paper and responding to reviewers' comments.

Papers Not Included in the Thesis

I. Screening of Nuclease Activity in HNSCC using Oligonucleotide Probes: towards a novel functional Biomarker of Treatment Response in Head and Neck Cancer
Balian A., Akhtar KT., Hernandez FJ., Roberg K., Hernandez LI. *In Manuscript*

Author's contribution: AB has equally contributed to the experimental work along with a co-author in the paper. AB reported, plotted and discussed data and contributed to writing the first draft.

Conference Contributions

- Soma Probes Symposium, San Sebastian, Spain, 2021 (oral presentation)
- The International Round Table on Nucleosides, Nucleotides and Nucleic Acids, Stockholm, Sweden, 2022 (scientific poster presentation)
- Oligonucleotide Therapeutics Society (OTS) annual meeting, Phoenix, Arizona, USA 2022 (scientific poster presentation).

  OTS travel grant for the annual meeting in Phoenix, Arizona, USA 2022. Best poster award OTS annual meeting 2022

List of Abbreviations

AMPs	antimicrobial proteins and peptides
ANG	angiogenin
APE1	apurinic/apyrimidinic endonuclease 1
BRCA1	breast cancer gene 1
BRCA2	breast cancer gene 2
CF	cystic fibrosis
CIN	cervical intraepithelial neoplasia
CPG	commonly controlled pore glass
DFS	disease-free survival
DLAD	DNase 2-like acid DNase
DMT	4,4´-dimethoxytrityl
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGTA	Ethyleneglycoltetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
exRNA	extracellular RNA
FAM	fluorescein amidite
FDA	Food and Drug Administration
FEN1	Flap endonuclease-1
FRET	Förster resonance energy transfer
FSH	follicle-stimulating hormone
GPI	glycosylphosphatidylinositol
HBV	hepatitis B virus
hcRNases	Human canonical RNases
HER2	human epidermal growth factor receptor 2
HIV	human immunodeficiency virus
HNSCC	head and neck squamous cell carcinoma
HPLC	high-performance liquid chromatography
HSV-1	Herpes simplex virus 1

HTS	high-throughput screening
HUVS	hypocomplementemic urticarial vasculitis syndrome
IBD	inflammatory bowel diseases
IHC	Immunohistochemistry
IRE1α	inositol-requiring transmembrane kinase endoribonuclease-1α
LH	luteinizing hormone
mEar	mouse eosinophil-associated ribonuclease
MRE11	meiotic recombination 11
MRI	magnetic resonance imaging
NETs	neutrophil extracellular traps
NMR	nuclear magnetic resonance
NM23-H1	Non-metastatic protein 23 H1
nsp14-ExoN	NSP14 proofreading exoribonuclease (ExoN)
NST	no special type
PA	polymerase acidic protein
PDE	phosphodiesterase I
RA	rheumatoid arthritis
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
RSV	respiratory syncytial virus
RT	reverse transcriptase
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
SPION	superparamagnetic iron oxide nanoparticles

Table of Contents

Chapter 1

1.Introduction

1.1 Background

Nucleases are a group of enzymes that exert catalytic activity on deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and chemically modified oligonucleotides. These enzymes hydrolyze the phosphodiester bonds that connect between the sugar moieties of nucleic acids. Thus, nucleases function as biological scissors that possess a shared catalytic capability, but exhibit a large variation in structure, function and substrate selectivity[1]. Nucleases play crucial roles in nature, including survival, homeostasis [2-6] and immune defense [3, 4, 7-10]. They are involved in genome stability [11], genome editing and proof-reading [12], DNA repair [13] and cell apoptosis [14-18]. Nucleases are also key elements in host defense against pathogens [10, 19] and innate immunity modulation during infection and cancer [20, 21]. Some nucleases exert neurotoxicity and angiogenesis [3, 4].

Nucleases are categorized into DNases that cleave DNA or RNases that cleave RNA or can be both types in one. Endonucleases excise internally, while exonucleases cut from one end of nucleic acid. Some nucleases are both in one. Nucleases are single stranded (ss)- or double stranded (ds) nucleic acid specific or break down both. Some nucleases cleave DNA recognizing a specific sequence (restriction enzymes) [1]. Moreover, nucleases are naturally occurring enzymes in all of the three domains of life: Bacteria, Archaea, and Eukarya [22-24], as intracellular [25], membrane bound [25, 26] or secreted proteins [3]. The diversity of these enzymes encouraged implementation in wide applications, as tools in nucleic acid bioengineering [27], therapeutic molecular targets [28-34], therapeutic agents [2, 25, 35], and as disease biomarkers [36, 37].

Given their physiologic implication, it is not surprising that alteration in nucleases could be indicative of alteration in human conditions, hence they could be utilized as biomarkers. Indeed, a plethora of studies have reported alteration in nuclease expression and/or activity in correlation with several diseases. The list includes autoimmune diseases such as systemic lupus erythematosus (SLE) [26, 38-40] and

hypocomplementemic urticarial vasculitis syndrome (HUVS) [41]. Asthma, obesity [42] [43], cataract [44-46], cardiovascular diseases [47-50], schizophrenia [51] and infectious diseases [37] are other examples. Importantly, nucleases have caught research interest as potential biomarkers for cancer diagnosis. It is well established that early cancer detection is an essential requirement for successful treatment. The pursuit remains ongoing as most clinically available biomarkers are either not sufficiently accurate when used alone or are not present at early stages, as in the case of breast cancer biomarkers [52, 53]. Nucleases are promising biomolecules in this sense. Multiple nucleases display alteration in expression and activity in correlation with cancer as reported in research and summarized in paper I [36]. Nucleases were even suggested to promote cancer, such as DNase4 in prostate cancer [54] and RNase5 in many cancers [55-60]. Other studies indicate a tumor suppressor role of nucleases [61]. Some nucleases display a dual role depending on the site or organ in question [62] [63].

Furthermore, DNase activity was described back in 1800s. It was first noticed in bovine organs, which warranted the characterization of the proteins exhibiting nucleolytic activity [6]. Despite this ancient discovery of their catalytic activity, nucleases were mostly studied at the protein and mRNA level in research, including cancer research. Harnessing nuclease activity as a biomarker in cancer diagnosis was relatively less applied. Indeed, there are fragmentary studies with sometimes inconsistent results regarding nucleases activity in respect to diseases. This reflects the insufficiency of the methodologies applied and the lack of a solid unified approach to report correct results [64].

Based on the body of knowledge, demonstrating the value of nucleases as cancer diagnostic biomarkers, and the availability of nucleic acid probes, we were prompted to investigate the utility of cancer-associated nuclease activity as diagnostic biomarker. To detect this activity, proper tools are required. Among these tools are nucleic acid probes, also referred to as oligonucleotides, of our design. The oligonucleotides are designed to be cleaved by cancer nucleases but resistant to healthy or endogenous nucleases. The probe selection is performed through several screening rounds. In the first round an initial library of natural and chemically modified oligonucleotides are used as substrates. Based on the sequence/s of the promising probes, in that the ones most

discriminating between healthy and cancer, a next generation of probes is designed with improved sensitivity and specificity. At the end of an iterative process of try and error, the best probes are selected. The protocol of probe design, screening for nuclease activity and probe selection is described in the paper II [65].

Nucleases represent a novel possibility in breast cancer diagnosis, for which more specific and sensitive biomarkers than those currently available are still an unmet clinical need [66]. This by utilizing the catalytic activity as biomarker. Given that nuclease activity profile associated with breast cancer differs from the one in healthy status, breast cancer can be diagnosed using the "suitable" substrate for nucleases. The substrates should be sensitive to and specifically cleaved by the cancer associated nucleases. We and others designed and used nucleic acid probes/ oligonucleotides of 8-12 nucleotides as substrates to screen for nuclease activity associated with a disease [67-70]. The probes in this work, except for those in the paper V, are flanked with a reporter and a quencher, and a standard fluorescence plate reader is used for acquiring the signal. The probes used in this work are mainly FRET-probes. On the 5'-end FAM (fluorescein amidite) fluorophore is coupled to each oligonucleotide, while on the 3'-end the quencher TQ2 (tide quencher 2) is attached. Initially a probe is at "off "state, meaning that any signal from the fluorophore is quenched by the proximately existing quencher on the same probe. When the probe is degraded by nucleases, this proximity is disrupted, and the signal emitted by the fluorophore is detected by a plate reader. Thus, nuclease activity is quantified and reported in terms of fluorescence intensity.
In this thesis, nuclease activity is investigated as a potential biomarker in breast cancer diagnosis utilizing a panel of nucleic acid probes as substrates. A solid protocol for nuclease activity screening, nucleic acid probes design and tailoring is utilized to generate a modulable, sensitive and specific detection platform with easy translation into clinical use. The **Figure 1** provides a schematic representation of screening for nuclease activity and design of oligonucleotides that are specific substrates for cancer-associated nucleases.

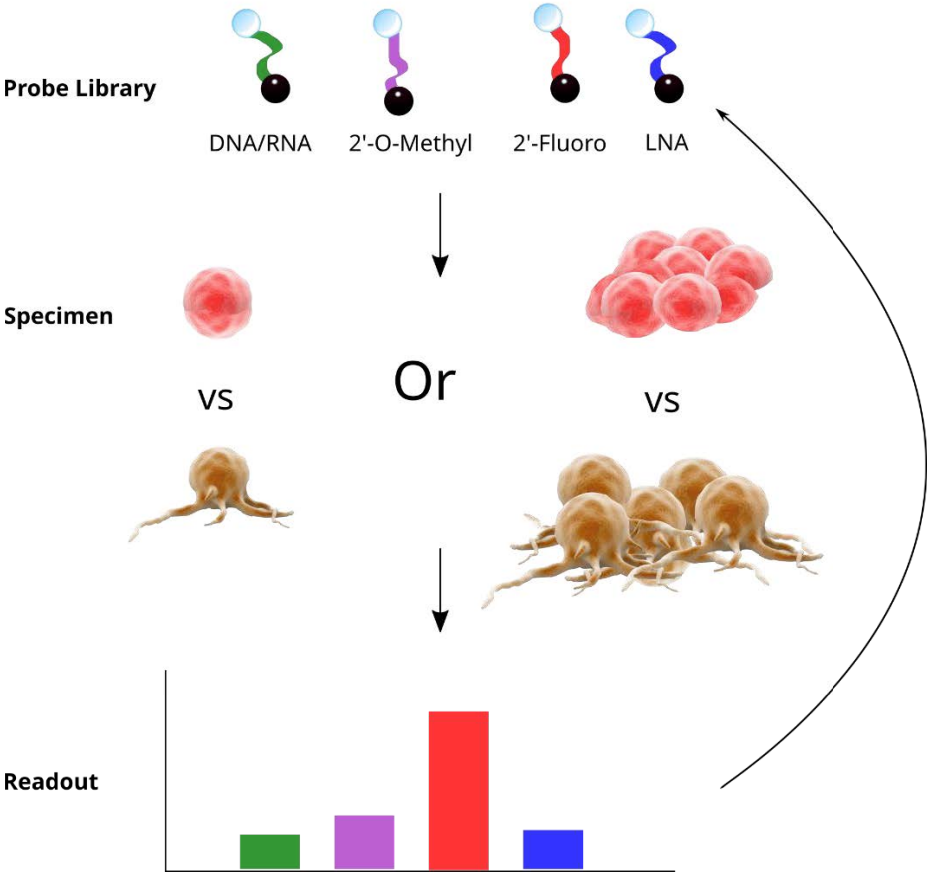

Figure 1. A schematic representation of screening for nuclease activity and probe selection. Screening for nuclease activity is performed in rounds, using a library of natural or chemically modified probes as substrates, and the best performing probes are selected.

1.2 Objectives

The aim of the thesis is to develop non-invasive diagnostic methods based on nuclease activity associated with cancer as a biomarker and nucleic acid probes as substrates. This global aim is distributed into the following sub-aims that each paper tackles.

In paper I, we have aimed to summarize literature findings on nucleases as potential biomarkers in cancer diagnosis, in a review article. Additionally, we shed a light on the utility of cancer-associated nuclease activity as a promising biomarker for cancer diagnosis, using nucleic acid probes as substrates.

In paper II, we have aimed to describe a method of screening for nuclease activity that cleaves substrates of nucleic acid probes to differentiate between disease condition and healthy. We explain how probes are tailored to be sensitively and specifically degraded by disease nucleases, based on iterative rounds of screening and probe design.

We have sought, in paper III, to explore nuclease activity profile of breast cancer cell lines. Breast cancer cells along with healthy counterparts were screened for nuclease activity, utilizing a library of 75 oligonucleotides as substrates.

The aim of the study presented in the paper IV is to proof the concept of the utility of nuclease activity associated with breast cancer tumors as a biomarker to discriminate between cancer and healthy. We have attempted to identify nucleic acid probes that discriminate between clinical samples of breast cancer and healthy tissues with high accuracy, sensitivity, and specificity.

In the study presented in paper V, we aimed to develop activatable magnetic resonance imaging (MRI) probe for detecting a specific nuclease, micrococcal nuclease (MN) secreted by *Staphylococcus aureus* (*S. aureus*). Thus, providing a step forward in the translation of harnessing nuclease activity as biomarker into the clinical use.

1.3 Thesis Outline

The thesis begins with an introductory part in which an overview, the general aim, and the outline of the thesis are discussed. The chapter 2 details nucleases, providing a glimpse on the evolution of nucleases. In chapter 3, the applications of nucleases in the biomedical field, mainly in diagnosis are discussed. Chapter 4 is specified for cancer associated nucleases and nuclease activity as a potential cancer diagnostic biomarker. Information on breast anatomy and breast cancer types and diagnosis is summarized in chapter 5. Chapter 6 comments on the methods used in this project. Chapter 7 provides a summary on each paper included in the thesis. Key results from each paper are summarized in chapter 8. In chapter 9, the conclusion of this work is stated followed by the future directions in the chapter 10.

Chapter 2

2. Nucleases

2.1 Definition and Characteristics

Nucleases are enzymes that hydrolyze the phosphodiester bonds in the sugar moieties of nucleic acids and oligonucleotides [1]. The phosphodiester bond is the bond with highest stability in biology, adding up to the uniqueness of these enzymes [71]. Nucleases recognize, bind and cleave nucleic acids [72]. They share the catalytic capability, but also exhibit a large variation in structure, function and substrate selectivity. These enzymes might show similar structure and differ in function, or they might overlap in physiological roles or cleavage patterns despite of different structure [1]. DNase activity was reported since 1800 [6]. And the description of nuclease activity identified as enzymatic cleavage of nucleic acids was described 1903 [73]. About four decades later, nucleases were classified according to their substrate sugar preference into (deoxyribonucleases) DNases and (ribonucleases) RNases that hydrolyze DNA and RNA, respectively [74]. Studies revealed later that nucleases were phosphodiesterases [72]. The sugar specificity has been an essential property in the classification of nucleases [72]. However, some nucleases display no sugar preference and digest both DNA and RNA sufficiently. Nucleases may vary in catalysis preference towards single or double-stranded substrates, and some nucleases exhibit a sequence specificity [1].

Nucleases can be divided into categories based on different characteristics including their substrate cleavage properties and/or their biological functions, their biochemical mechanisms, or their sequences, fold and active site structure [75]. Indeed, many attempts were done to classify nucleases [76-78]. To refine the classification, adapting consensus criteria was suggested several decades ago. The criteria encompass; 1) the nature of hydrolyzed substrate (DNA or RNA), 2) the type of the nuclease cleavage (endonuclease or exonuclease), 3)the nature of the reaction products mono- or oligonucleotides with 5'- phosphate or 3'-phosphate ends and 4) the nature of the hydrolyzed bonds (Pu ↓ Py, Py ↓ Pu) [79]. Although this system was more inclusive than the previous ones, strand specificity, site specificity and nature of the substrate such

as damaged DNA were emerging traits that were not included. Moreover, nucleases with multifunctional and diverse characteristics did not fall into a specific category [72, 80]. To overcome this, a classification system was proposed by Linn [81]. The classification is shown in the **Figure 2** [80].

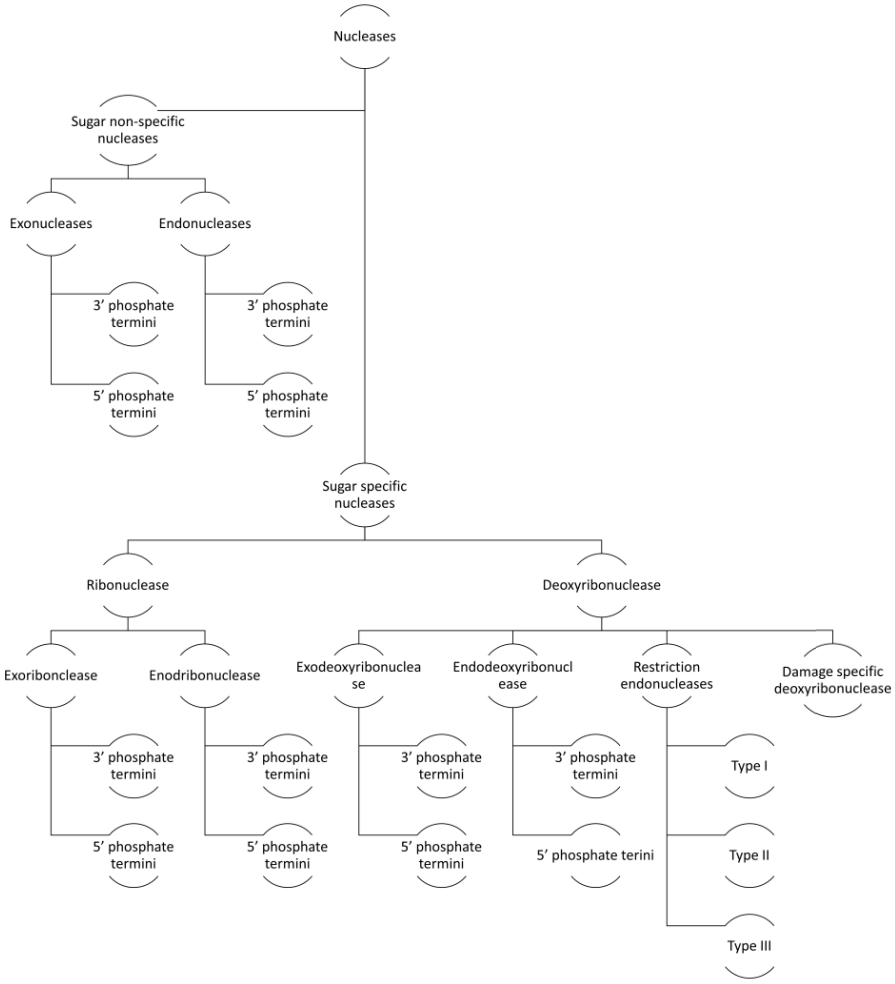

Figure 2. One of the classification systems of nucleases. Nucleases are classified based on substrate sugar preference, cleavage site and sequence specificity.

Furthermore, exonucleases break down at one end of a nucleic acid and clip out one nucleotide a time. They are subdivided into two categories:

one cutting from 5' to 3' and the other from3' to 5', and some are able to degrade DNA in both directions [1, 12]. Majority of dsDNA specific exonucleases process only one strand, and therefore exhibit a specific polarity either to 5' (cutting in 5′– 3′ direction) or 3' end (cutting in 3′–5′ direction) [12, 23]. Likewise, most ssDNA degrading exonucleases show a specific polarity [23]. A 3' to 5' exonucleolytic activity can be associated with DNA polymerase, intrinsic or autonomous [12]. DNA Polymerases maintain the genome integrity during DNA repair, recombination, and replication. The combined exonuclease activity acts as a proofreader that improves the accuracy of polymerases in DNA synthesis by cutting out nucleotides that have been erroneously incorporated during polymerization [12]. Exonuclease activity is performed by an exonuclease domain other than a polymerase domain in this associated type [23]. Additionally, 3'– 5' exonucleases can be autonomous proofreaders that are not associated with polymerase. Three Prime Repair Exonuclease 1(TREX1) and Three Prime Repair Exonuclease 2 (TREX2) are examples of the independent autonomous proofreading 3'– 5' exonucleases [12]. Proofreading exonucleases have 3'– 5' polarity, however not all 3'– 5' exonucleases have proofreading function. For instance, the apurinic/apyrimidinic (AP) endonuclease (APE1) and meiotic recombination 11 (MRE11) (also known as Double-strand break repair protein MRE11) both display 3'– 5' exonuclease activity. These two nucleases contribute to base excision repair and double-strand break repair, respectively [12]. Most of DNA exonucleases do not cleave RNA, however some degrade both nucleic acids such as RNaseT [23]. The cleavage products of exonucleases are either mononucleotides or oligonucleotides [1, 23]. Some exonucleases excise one nucleotide per substrate binding event and are termed distributive, while others achieve multi hydrolyses and are known as processive [12, 23].

Endonucleases degrade within a nucleic acid, generating oligonucleotide products [1]. The degradation is either general or sequence specific as in the case of restriction endonucleases [1, 12], with a preference towards ssDNA or dsDNA or without [12], and targets DNA or RNA [24]. Restriction endonucleases cleave dsDNA upon recognition of a sequence in a highly specific manner. This DNA sequence is termed cutting or recognition site that may often include four, six or eight nucleotides. Substitution of one of these nucleotides renders the recognition site into

resistant to the respective restriction enzyme [82]. This specificity of cleavage rendered restriction endonucleases to valuable tools in biotechnology that initiated the era of recombinant DNA research [27]. Endo- and exonucleases possess a shared chemistry of degradation, therefor some nucleases act as both endo- and exonucleases recruiting the same active site [12]. Some nucleases of the family Flap endonuclease 1 (FEN1), for example, have both endonucleolytic and 5'– 3' exonucleolytic activity. MRE11 degrades dsDNA as a 3′–5′ exonuclease while cleave ssDNA as endonuclease [1, 12]. Nucleases are expressed intracellularly such as Flap endonuclease-1 (FEN1) [83], or secreted to the extracellular matrix such as the human canonical RNases [3] and most of bacterial nucleases [2-4], or are cell membrane bound such as DNase1L1 [26].

The physiological roles of an enzyme are mainly defined by comparison to relevant mutants [72]. Initially, it was thought that the main role of nucleases is the metabolism of nucleic acid in the salvage pathway of nucleic acids [72]. Then, pivotal functions of nucleases in wide spectrum of organisms were unraveled. Globally, nucleases are one of the cellular machineries for clearing of nucleic acids, either those that exceed the biological requirements of the cell or the exogenous nucleic acids of pathogenic source [84]. Microorganisms use nucleases for DNA replication, recombination, nucleic acids digestion, apoptosis and facilitating the adhesion to host cells. A variety of exo-, endo and restriction nuclease are involved in these processes. For example, bacterial secreted nucleases mediate escaping host immunity, degradation of neutrophil extracellular traps (NETs) and biofilm modulation. Hence, they are considered as bacterial virulence factors [2-4].

Viral nucleases are key players in virus replication and virus–host interactions. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) NSP15 endoribonuclease also known as EndoU contributes to evading host immune system [28]. NSP14 proofreading exoribonuclease (ExoN) (nsp14-ExoN) is essential for proof-reading during genome transcription and replication of SARS-CoV-2 [29]. RNaseH domain in reverse transcriptase (RT) of human immunodeficiency virus (HIV) [85, 86] and hepatitis B virus (HBV) [31] is essential for viral genome replication. This enzyme catalyzes hydrolysis of RNA in DNA·RNA intermediate heteroduplexes constructed of viral RNA and host DNA

during viral replication, allowing DNA to be recruited in a new viral replication cycle [31, 85, 86]. The polymerase acidic protein (PA) in RNA polymerase of Influenza virus A exhibit endonuclease activity that is crucial for the viral transcription and replication [87]. Moreover, genome packaging in Herpes simplex virus 1 (HSV-1) is achieved by terminase endonuclease [88].

In human body, nucleases are abundantly expressed. They contribute to host-defense activities [10, 19]. The human canonical RNases, for instance, play crucial roles as immune hos defense factors [3, 19]. Nucleases in human body are also involved in innate immunity modulation during infection and cancer [20, 21], DNA repair [13], genome stability [11] and cell apoptosis [14, 18, 89]. Additionally, homeostasis of DNA concentration is attributed to DNases that metabolize DNA. This function is immensely important as accumulated non-digested DNA in blood leads to formation of anti-DNA autoantibodies that build antibody-DNA complexes, triggering an ensemble of autoimmune diseases [15, 26].

2.2 The Basic Chemistry of Nuclease Catalytic Reaction

Nucleases are phosphodiesterase enzymes that hydrolyze the phosphodiester bonds linking between the sugar moieties in nucleic acids, by cleaving either the scissile bond P-O 3’ or P-O 5’, as shown in the **Figure 3** [1, 72]. The products are either 5’-phosphate and 3’-OH, or 3’-phosphate and 5’-OH , respectively [1, 72]. This mechanism of catalysis is performed by ribozyme and protein nucleases in contrast to chemzymes that cleave nucleic acids by disrupting sugar molecules via oxidation or alkylation of bases [72].

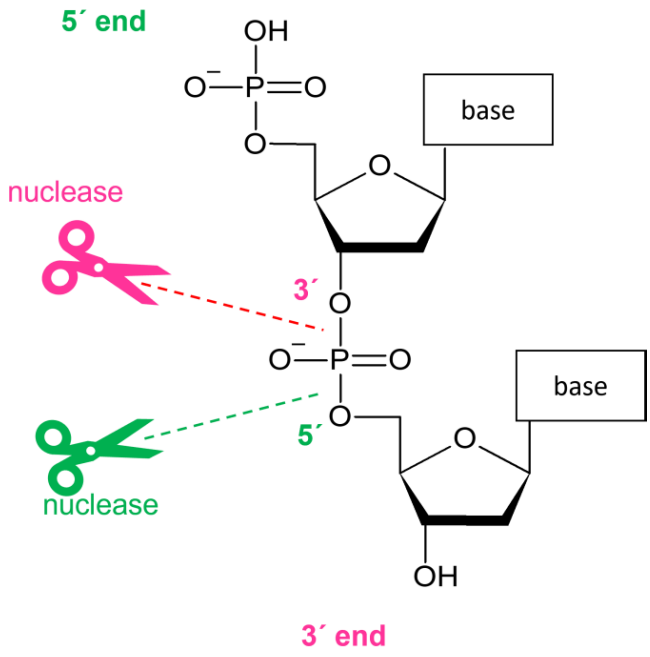

Figure 3. A simplified schematic representation of cleavage of phosphodiester bonds by nucleases. Nucleases can cleave the scissile bond P-O 3' or P-O 5'.

The cleavage reaction is considered as a general base-acid reaction of S_N2 type that is completed in three steps. First, a nucleophilic attack takes place. The nucleophile should be on the 5'side poised for in-line attack to break p-O 3' bond, which results in 5'-phosphate and 3'-OH products. Deprotonated water molecules are the most common nucleophiles. DNA or RNA hydroxyl group of the 3'end could also serve as nucleophile. The side chains of Serine (Ser), Tyrosine (Tyr) and Histidine (His) might be utilized by DNases for this purpose and the 2'-hydroxyl group of RNA is utilized by RNases. The position of the nucleophile is on the 3' side to cleave the P-O 5' bond. At the second step, a highly negatively charged penta-covalent intermediate is formed . At the third and last step the scissile bond is degraded [1]. Some amino acid residues in a nuclease structure play key role in the activation of water molecule that attacks the sciccile bond, stability of the intermediate compound and departing of the leaving group [90]. Enzymological and mutagenesis studies besides X-ray crystallography and chemically modified substrate use, among others, enabled understanding nuclease-nucleic acid interaction [75, 90, 91].

The dominant, and likely preferable, type of cleavage is that generating products with 5'-phosphates and 3'-OH groups. This could be attributed to the fact that 3'-OH can be recycled (utilized) by other nucleic acid processing enzymes such as DNA ligases as well as RNA and DNA polymerases. Additionally, 5'-phosphates are material for DNA ligation in DNA repair, recombination and replication pathways [1].
Majority of nucleases recruits divalent cations as cofactors for the catalytic activity, especially magnesium Mg^{2+} and calcium Ca^{2+} that are abundantly available in the body. Other cations that function as cofactors are iron Fe^{2+}, zinc Zn^{2+}, manganese Mn^{2+} and copper Cu^{2+} [1]. In metal ion-dependent nucleases, metal ions deprotonate water molecules facilitating a nucleophilic attack by the oxygen [91]. The metal ions also facilitate the phosphoryl transfer reactions by stabilizing the intermediate compounds [92]. Some nucleases recruit oxygen of an inorganic phosphate group as a nucleophile that attack the scissile bond. Metal ions are important for the catalytic activity of these nucleases, however the exact role of metals in such a reaction remains to be explored [91]. Some nucleases, however, are metal-ion-independent [1]. In ion independent RNases, the active site residues activate the 2'-OH oxygen by deprotonation achieved by displaying required pKa values [91].

2.3 Hint on Evolution of Nucleases

The evolution of nucleases is reflected by the wide diversity they express in different organisms. Similarity in function and homology in structure might coexist in nucleases, same biological event could be achieved by different nuclease across organisms, or same nuclease is assigned to different functions in different organisms [1]. For example, bacterial nucleases are virulence tools [2-4], while in human some nucleases are host defense arsenal [3, 7, 10]. Some nucleases exhibit an interspecies conserved amino acid sequence such as RNase4, hinting on a unique physiological role [54].

Enzymes in general are classified into families and superfamilies based on similarities in structure and function. Alteration in the protein sequence might result in an alteration in function [93]. However, the same reaction could be catalyzed by different enzymes in general [93], likewise the same biological function could be carried out by different nucleases

[1]. Evolution of enzymes is chemistry-driven or substrate driven [93] that exemplifies the ability of nature to endure adapting to numerous changes [94]. In fact, this property has been exploited by researchers through applying directed evolution to generate enzymes with novel and beneficial catalytic functions [94].

A good example on nuclease evolution is RNase2 and RNase3 also known as eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP), respectively [8]. The genes encoding these nucleases were identified as two of the most rapid evolving genes in primates [95], and evolution of RNase3 was attributed to directional positive selection [95]. These nucleases are among the main proteins secreted by eosinophiles as they exert an antimicrobial activity. They belong to the human canonical RNases (hcRNases) that are vertebrate exclusive nucleases [8]. It was suggested that this family has a host defense ancestral origin from which it evolved, as antibacterial activity was observed for the members with distant relation to the ancestral RNases [96]. Another suggestion is that these RNases started off as proteins with angiogenesis function [3].

The hybridizing sequences were detected in primates but not in non-primate mammals, suggesting a high divergence. A single predecessor for EDN/ECP with a low nucleolytic activity was identified in the New World monkeys. Thus, it was postulated that these genes originated from a duplication that took place following divergence of the New World from the Old-World monkeys. After the duplication, the genes had accumulated non-silent mutations at higher rates than other functional coding sequences in primates. However, the amino acid sequences essential for nuclease activity were conserved [7]. The rapid divergence of RNase2 and RNase3 hints on a response to unusual evolutionary pressure [97], that is thought to be related to their host defense roles [7]. A full elucidation of these evolutionary constraints remains to be achieved [98].
This divergence is hypothesized to promote antipathogen functions of these proteins [98] .RNase3, in the range of micromolar concentration, showed toxicity against schistosomula, trypanosomes and other human parasites in vitro. It was also active against Gram-negative and Gram-positive bacteria *in vitro*. RNase2 is an early activator of immune responses, both innate and adaptive types [8]. It has no antibacterial activity but displays an antiviral activity on RNA-virus such as HIV and

respiratory syncytial virus (RSV) [3]. The antimicrobial activity of RNase 3 is not dependent on its nucleolytic activity but is attributed to its ability to disturb the bacterial membrane. However, RNase2 antiviral activity is linked to its ribonucleolytic activity [3, 8].

The mouse eosinophil-associated ribonuclease (mEar) −1 and −2 were the first rodent orthologs of RNase2/RNase3 identified. This was followed by identification of several mEars in mouse and rat. These proteins diverge from the human orthologs, possessing only 50% homology. They are also evolving at a high rate under positive selection pressure. More about the function of rodent orthologs remain to be elucidated. It was observed, however, that some such as mEar11 and mEar6 are expressed in response to infection. Hence, the sequence diversity and ribonucleolytic activity were the two pillars for understanding the function of RNase2 and 3 and the Ears, mainly a proposed function in host defense against virus pathogens [98].

2.4 Human Nucleases

Nucleases are widely expressed in organisms, offering variety in sugar preference, endo- and exonucleolytic activity, metal ion dependence, structure and function [1]. Besides, emerging nucleic acid hydrolyzing enzymes are constantly added to the list [71]. Therefore, providing a full list of nucleases in human is not straight forward. This section discusses an assembly of DNase1, 2 and 3 in addition to secretory RNases in human. These DNases [71] and RNases [3, 19] have occupied a central interest of research for their potential applications in diagnosis and therapy. Herein, the nucleases presented are categorized according to their sugar preference to facilitate classification.

DNases

DNases are essential enzymes in maintaining human health [99]. DNases degrade the phosphodiester bonds in DNA molecules. Extracellular DNA (ecDNA) is present as a consequence of apoptosis or active secretion by cells. ecDNA along with the bound proteins activate a cascade of immune and inflammatory responses, mainly through activation of DNA sensing receptors expressed on both sides of a cell membrane. Indeed, high concentration of DNA in plasma is associated with several inflammatory diseases. Therefore, DNase nucleolytic activity is crucial for maintaining

an optimal physiological concentration of DNA [25]. DNases have evolved in human along with the phagocytosis to aid in bacterial DNA degradation according to the most prominent hypothesis [100]. Also, DNases have evolved as a protection machinery that degrades self-DNA intra- or extracellularly under physiological or pathological condition, preventing its harmful accumulation [99]. There are two families of DNases that differ in biochemical characteristics. The first, DNase1, consists of DNase1, DNase1L1, DNase1L2 and DNase1L3. The second family, DNase2, comprises DNase2α, DNase2β and L-DNase2. The gene of the latter however is not DNase but Serpin Family B Member 1 (SERPINB1). TREX1 that is included in this section does not belong to these families of DNases [25].

DNase1 is the first and one of the most comprehensively characterized members of the DNase1 family, with the catalytic site mechanism of action fully elucidated. This enzyme belongs to the mammalian DNase1 family [71]. It degrades DNA optimally at neutral pH and generates products of 5'-end phosphate [6]. Degradation towards dsDNA is more efficient than that towards ssDNA, with the B-form being the most adequate structure [25]. The cations Ca^{2+} or Mg^{2+} are required for the nucleolytic activity of DNase1 [71]. Chelators such as Ethylenediaminetetraacetic acid (EDTA) or Ethyleneglycoltetraacetic acid (EGTA) inhibit the catalytic activity. G-actin is a natural potent and specific inhibitor of DNase1 [71]. DNase1 in human is of the pancreatic type, referring to the organ in which it is expressed. The enzyme is secreted to the intestinal lumen. It is pronouncedly present in bodily fluids including plasma where it clears ecDNA and thereby prevents autoimmune reactions [25, 64]. It was suggested that this role is achieved along with DNase1L3 [25]. DNase1 is one of the main DNases in blood, however only a proportion of it is in the active form in blood. The highest nucleolytic activity is detected in urine [101].

DNase1L1 (DNase X/Xib), DNase1L2 and DNase1L3 (DNase γ) are known as DNase1-like nucleases due to the similarity with DNase1 in amino acid sequence and nucleolytic characteristics [71, 102]. They degrade DNA to generate products with 5'-phospho and 3'-hydroxy ends. These enzymes need Ca^{2+} or Mg^{2+} as cofactors and are inhibited by Zn^{2+}, but unlike DNase1 they are not inhibited by actin [25, 71]. The pH optimum is neutral for these DNases except for DNase1L2 that is active

optimally at acidic pH. Expression of these enzymes originates from different organs and tissues. DNase1L1 is expressed in skeletal muscles and cardiomyocytes. DNase1L2 is abundantly expressed in keratinocytes of the stratum corneum where it cleaves the nucleic acids in bacterial biofilms. And lower levels are present in several tissues such as lung, brain, and placenta. DNase1L3 is expressed in lymphoid organs. DNase1L2 and DNase1L3 are present extracellularly, however the secretion from the tissues of origin remains to be understood [25]. DNase1L1 is expressed in the cytoplasm with no secretion being detected [25]. This is attributed to its glycosylphosphatidylinositol (GPI) anchor located in the C-terminus. Thus, it is anchored to the cell membrane with the active site outside the cell, which allows an extracellular nuclease activity [26]. It was suggested that DNase1L1 acts as a guardian of the cell against integrating a foreign gene [102]. The DNase1-like nucleases cleave likely chromatin DNA upon apoptosis [25], and DNase1L1 cleaves naked DNA. DNase1L3 maintains ecDNA homeostasis in plasma. However, a specific biological role of these DNases has not been confirmed so far [25, 26].

DNase2 family members are active at an acidic pH, hence they were previously named acidic DNases. The first characterized member was denominated DNase2, but when a homolog was isolated the name was changed into DNase2α while the homolog was named DNase2β or DNase2-like acid DNase (DLAD) [25]. Unlike DNase1 family, DNase2 is ion-independent and produces molecules with 5'-hydroxy and 3'-phospho ends by cleaving DNA [71]. DNase2 is secreted to the bodily fluids such as blood and urine, with generally low concentrations detected [71]. And the nuclease activity is widely present in various tissues [6].
DNase2α is an endonuclease that hydrolyzes DNA non-specifically. The activity is greater towards dsDNA compared with ssDNA. At a neutral pH, the catalytic activity is significantly decreased. DNase activity is also decreased by divalent cations, primarily by Fe^{2+} add Cu^{2+} and milder decrease is resulted by Mn^{2+}, Mg^{2+} and Ca^{2+} and Zn^{2+}. NaCl inhibits the activity as well. This enzyme is thermally resistant, at least at 60ºC, as the total activity inhibition is observed at 75°C. It is located in the lysosomes intracellularly, but the enzyme is also secreted to the physiological fluids such as blood, urine and saliva with small amounts [71]. The acidic pH optimum and presence in lysosomes define digestive and protective roles for this DNase. For instance, it protects the skin

against bacteria and virus, and along with DNase1L2 it functions as a shield for skin [25]. It was suggested that it hydrolyzes DNA in nucleosome following cell apoptosis and phagocytosis. DNase2α does not participate in DNA replication, recombination or repair because it produces cleavage products with 3'-phospho ends [71].

DNase2β is a homolog to the DNase2α with 37% shared identity and similar catalytic fashion. The genes encoding both proteins have different chromosomal locations. DNase2β is expressed in salivary glands and with lower levels in lungs and prostate [71]. This enzyme is functional in acidic pH. Among divalent cations, Zn^{2+} is the strongest inhibitor of the catalytic activity that is also slightly inhibited by Co^{2+} and Ni^{2+} [103]. The biological role of DNase2β is not fully clear. However, it was demonstrated that it cleaves nuclear DNA upon differentiation of fiber cells in the lens preventing cataracts that is resulted in by the accumulation of non-degraded DNA [25].

L-DNase2 is active at the same conditions as for the other members; acidic pH and without cations. This DNase is encoded by the gene *SERPINB1* the initial product of which is Leukocyte Elastase Inhibitor, an anti-protease protein. This protein provides protection to the cells upon the course of inflammation. Upon long exposure the amino acid sequence is truncated, and the protein is translocated from the cytoplasm to the nucleus where it acquires endonuclease activity. This latter form is known as the L-DNase2, which contributes to degradation of DNA in apoptotic cells. However, to decipher the physiological roles more research is required [25].

DNase3/TREX1 and TREX2 are 3'-to-5' exonuclease that degrade both ds- and ssDNA, with a preference towards the latter by TREX1 [104]. TREX1 was first identified when a distinct DNase activity was discovered in nuclei of mammalian cell and was denominated as DNase3 [105]. TREX2 is a homolog to TREX1, and both belong to the DNAQ-like exonuclease family [25, 104]. These exonucleases process DNA by removing nucleotides upon repair, recombination and replication, maintaining DNA integrity [104]. They are key players in cutaneous homeostasis, and aberration in their pathways have been linked to many conditions including SLE, Aicardi-Goutieres syndrome, psoriasis and skin cancer [104]. All cells express TREX1, mainly in the cytoplasm

[104]. TREX1 regulates immune response, and mutations have been correlated with a panel of autoimmune diseases, such as SLE. The enzyme digests endogenous DNA debris in cytoplasm. Non-degraded DNA activates DNA sensing pathways to trigger innate immunity against self-DNA, prominently through INF-1 activation [104]. TREX1 is employed by macrophages to clear DNA of intracellular NETs [105]. Besides, TREX1 is involved in HIV-1 processing [25, 105].
TREX2 expression is limited to tongue, skin, forestomach and esophagus. It is present in the cytosol but accumulates in nuclei of keratinocytes. This enzyme prevents tumorigenesis, through promoting cell death. It is capable of damaged nucleic DNA degradation, contributing to cell death through stimulating of an inflammatory response that clears dead cells [104].

RNases

The Human Canonical RNases

Since the RNaseA was isolated from the bovine pancreas over decades ago, enthusiasm increased towards identifying homologous RNases [4]. This led to the knowledge on the RNaseA superfamily, a vertebrate-specific enzymes family, the prototype of which is RNaseA [4, 106]. This family features unique characteristics and functions compared with other RNases. In human, 8 RNases belonging to RNaseA superfamily were identified and termed as the human canonical RNases [4, 107]. These 8 RNases are named RNase1-8, they are all secreted and the genes of which are located in the chromosome 14 within the cluster 14q11.2 [4]. The human canonical RNases function as an arsenal of our innate immunity, attributed to their role in host defense against pathogens [107]. In particular, RNase2,3,6,7 and 8 have reported antimicrobial activities [20]. These enzymes exhibit cytotoxic, antifungal, antibacterial and antiviral activity that is not necessarily correlated with the ribonucleolytic activity of these RNases. Together hcRNases are key regulators of the immune response and inflammation [107]. Not surprising, an upregulated expression of one or more of hcRNases is associated with one or several disease conditions [20]. As such, better understanding of the multiple aspects of these enzymes, including their catalytic activity, would help to understand their potential as diagnostic and therapeutic biomarkers. These RNases are briefly described here and reviewed in detail elsewhere [3, 4, 20].

RNase1 is the human homolog of the bovine pancreatic RNaseA, however, it is suggested that in human the main role is not digestive [108]. RNase1 is expressed at almost all the tissues, prominently at endothelial cells. It degrades dsRNA, 2',3'-cyclic nucleotides and DNA:RNA hybrid. Its ribonucleolytic activity towards dsRNA is the most powerful among the vertebrate RNaseA superfamily, due to structural features. It prefers poly C over poly U as a substrate, but also cleaves poly A, and the pH optimum for the catalytic activity is 8 [3]. RNase1 is present in biological fluids such as blood, urine, spinal fluid, amniotic fluid and serum. In the latter, RNase1 functions as an extracellular RNA (exRNA) scavenger. It was suggested that RNase1 responds in a non-specific manner to pathogenic RNA. Additionally, it regulates inflammatory and immune response pathways through stimulating the maturation of dendritic cells and release of cytokines and chemokines, among others. A fetus protective role was suggested for RNase1, as it was observed to inactivate HIV [3]. RNase1 is upregulated in an array of maladies, such as sepsis, psoriatic arthritis, SLE, interstitial cystitis and leishmaniasis [20].

RNase2 and RNase3 are among the main secretory proteins of the eosinophil granules. RNase2 or EDN contributes to eosinophile driven neurotoxicity [20]. Unlike RNase1, these enzymes don't cleave dsRNA, 2',3'-cyclic nucleotides or poly A. They have catalytic preference towards poly U over poly C, and the pH optimum is 6.5-7. RNase 2 and 3 are both very basic proteins and share a significant primary structure identity of roughly 70%, however they exhibit different physiological and ribonucleolytic activities [3].
Besides the main source of eosinophils, RNase2 is expressed in monocytes and neutrophils, but also in spleen, liver, placenta, kidney and found in urine [20]. This protein lacks antibacterial activity, but exerts an antiviral effect on ssRNA viruses, correlated with its catalytic activity [109]. It blocks the replication of HIV, hepatitis-B virus, and respiratory syncytial virus (RSV) [20, 109]. RSV stimulates eosinophil degranulation, and RNase2 is a clinical biomarker of post-RSV bronchiolitis [109]. The catalytic activity of RNase2 is required to conquer the RSV genome, but also the structural characteristics of the protein are key elements for its antiviral efficiency [109]. Additionally, RNase2 is classified as an alarmin that regulates innate as well as adaptive immunity, solely through maturation of dendritic cells [3].

RNase3 is named eosinophil cationic protein (ECP), referring to its highly cationic nature [110]. The protein inhibits mammalian cell growth and has an antiviral and antiparasitic activity. A unique feature of RNase3 is its antibacterial activity, that is attributed to the capability of binding to and subsequently disrupting bacterial cell membrane. This binding mechanism is enabled by aromatic domains on the surface and the highly cationic nature of RNase3, providing high affinity to the bacteria cell membrane lipopolysaccharides [20]. RNase3 is dominantly expressed by eosinophils, but also neutrophiles and macrophages. RNase3 expression is upregulated upon infection. The secretion is stimulated by infection, inflammation and tissue injury when the eosinophils degranulate and release their protein content [110]. As such, the protein levels in the bodily fluids has been utilized as a diagnostic biomarker for eosinophil activation in inflammatory conditions such as asthma [20]. The antiviral activity of RNase3 against ssRNA viruses is correlated with the ribonucleolytic one [110]. In addition, RNase3 is capable of internalization of macrophages, where it abolishes intracellular infection in a manner dependent or independent on its ribonucleolytic activity [110].

RNase4 is a unique protein with the shortest sequence and greatest shared identity with its inter-species orthologs, among the hcRNases family members. It is abundantly present in nearly all the tissues with the highest levels in liver and lungs, which indicates a housekeeping role. Concerning the catalytic activity, RNase4 prefers poly U as a substrate over poly C, which is confirmed by structural studies. It hydrolyzes 2',3'-cyclic nucleotides, like RNase1. The physiological roles remain to gain consensus. The first role proposed was cellular RNA clearing. However, the clear preference towards poly U cleavage suggested a selective RNA recognition. Additionally, it was demonstrated that RNase4 and RNase5 have an anti-HIV activity [20].

RNase5 is a potent stimulator of neovascularization or angiogenesis and the first protein identified with an in vivo angiogenic effect [111]. It mediates rRNA transcription, a step required for other angiogenic factors to exert angiogenesis [112]. RNase5 was first isolated from serum-free supernatant of media conditioned with human colon adenocarcinoma cells HT-29 [113]. This RNase promotes tube formation in cell culture,

cell adhesion, proliferation, cell migration and invasion. RNase5 overexpression was correlated with many cancers [55-60], and it was suggested to be involved in cancer progression [60]. Studies have characterized ribonucleolytic active site in the protein, a cell binding site and a site for nuclear localization [112]. Both catalytic and noncatalytic sites are essential for the angiogenesis function. RNase5 exerts an antibacterial and antifungal activity as well [60].

RNase6 is the human ortholog of bovine kidney ribonuclease (RNaseK2), hereafter it was also named RNaseK6 [20]. The enzyme mRNA was detected in lung, liver, pancreas, kidney, placenta, brain, heart and skeletal muscle [3]. It was also detected in monocytes and neutrophils, suggesting a role in host defense [114]. The expression is upregulated upon genitourinary tract bacterial infections. RNase6 has a reported wide spectrum (gram positive and gram negative) antibacterial activity, but also an antiviral activity [4]. The remarkably lower ribonucleolytic activity towards yeast RNA compared with RNA2, suggests a low catalytic activity of the enzyme [3].

RNase7 was first purified from skin in an attempt to identify antimicrobial agents from healthy human skin [8]. It is mainly secreted by keratinocytes and has significant bactericidal effect on a panel of Gram-negative and Gram-positive bacteria. The nucleolytic activity is not essential for the bactericidal effect, that is linked to RNase7 capability of bacteria membrane permeabilization [3, 8]. RNase7 is expressed in epithelial cells of various tissues such as liver, kidney, respiratory tract, genitourinary tract, heart and skeletal muscle. It contributes to protection of epidermis and urinary tract against infections [3]. Additionally, it is one of the anti-microbial proteins and peptides (AMPs) in prenatal skin, which indicates a role in maintaining the sterility of amniotic cavity [20]. It is upregulated in correlation with sepsis, renal infection, dermal diseases such as atopic dermatitis and lesions of psoriasis, in addition to bacterial infections caused by among others *E.coli*, *Staphylococcus aureus (S. aureus)* and *Pseudomonas aeruginosa* [20]. On one hand, the upregulation is correlated with tissue injury in skin or even respiratory tract. On the other hand, RNase7 binds to self-DNA. As such, it was suggested that RNase7 activates immune response by detecting self-DNA resulted by cell death upon tissue damage [20].

RNase8 expression is limited to the placenta, spleen, lungs and testis. The physiological roles are governed by the exclusivity of expression, hence different from RNase7 despite of the large homology [115]. It has an antimicrobial activity on Gram-positive and gram-negative bacteria in addition to *Candida albicans*, which along with its expression in placenta indicates that it protects placenta from infections [3, 116]. Structurally, RNase8 has a glycine residue instead of cystine at position 84 that is conserved in all the other hcRNases. As it is the case for RNase 7, not much is known about the catalytic activity. It is worth to mention that no orthologs of RNase7 were found in the genome of mouse or rat [8]. It was suggested that RNase7 and the structurally similar RNase8 are the result of a recent gene duplication during evolution of primates [3].

More RNases were identified besides the 8 canonical ones. The RNase 9-13 share 15-30% identity with the 8 hcRNases, and structural differences were identified. Mainly, these enzymes have roles within the male reproduction system and seem to not require catalytic activity for their functions [20].
As such, the hcRNases are pivotal antimicrobial and immunomodulatory elements, the levels of which are altered in various pathological events. Therefore, investigating the nuclease activity of these RNases, and optimally selecting nucleic acid probe(s) as specific substrate for each member would open new avenues for diagnostic applications of several diseases or conditions.

Other RNases

RNaseT2 and RNaseH are other pivotal RNases in the human body. RNaseT2 in human is the only identified member of the RNase Rh/T2/S family. This family includes secreted endonucleases with wide abundance and high conservation in organisms from virus to mammals [117]. The enzyme in human is encoded by a gene located at the chromosomal region 6q27 [118]. The gene expression is detected almost in all tissues. Tissue injury and oxidative stress upregulate the expression [117]. The enzyme has intracellular and extracellular types. Intracellularly, RNaseT2 is found in cell organelles including vacuoles, mitochondria and lysosomes. It is suggested to contribute to mitochondrial RNA regulation as well as degradation of lysosomal RNA of endogenous or exogenous origin [117]. The secretory RNaseT2

exhibits an antimicrobial activity and immunoregulatory involvement which hints on a role in host defense [117]. Additionally, it is suggested to scavenge RNA in the extracellular matrix [20]. A catalytic feature might indicate a role in innate immunity regulation. RNaseT2 shows cleavage preference between GU or AU bases in ssRNA, the cleavage products are two nucleotide fragments with 2'3'-cyclic phosphate adenosine/guanosine terminus and uridine residue, respectively. By binding to the pockets of toll-like receptor 8 (TLR8), these molecules activate antipathogenic immune response [117].

RNaseH cleaves RNA in DNA-RNA heteroduplexes with as minimum as one ribonucleotide in a strand. The products are with 5'-phosphoryl and 3'-OH ends. Therefore, a suggested role of the enzyme is the removal of genomic ribonucleotides during DNA replication. The substrate-binding and catalytic motifs are located in the C terminus that is highly conserved. The N terminus of the protein contains a dsRNA-binding domain. Divalent cations such as Mn^{2+}, Mg^{2+} and Co^{2+} are required for the catalytic activity of the enzyme. And the pH optimum is 8-8.5 [119]. RNaseH mediated cleavage is exploited in antisense oligonucleotides (ASO) therapeutics used for target mRNA knockdown [120].

2.5 Oligonucleotides: Substrates of Nucleases

DNA and RNA consist of building blocks known as nucleotides [121]. Each nucleotide has three main components: a phosphate group, a sugar motif which is ribose or deoxyribose and a nitrogen base. Nitrogen bases adenine and guanine are purines, while thymine and cytosine are pyrimidines, and all of them are present in DNA. In RNA thymine is substituted with uracil, a pyrimidine. When the sugar molecule is ribose, the oligomer is RNA, while it is DNA when sugar moiety is deoxyribose. A nucleoside is a ribose or deoxyribose linked with a nitrogen bases through C-N glycosidic bond. A nucleotide is a nucleoside with a joined phosphate group to 5'-OH of the sugar moiety. An oligonucleotide is nucleotides linked to each other by a phosphodiester bond [121, 122]. The prefix oligo, of a Greek origin, means few or short referring to an oligonucleotide being a short sequence of nucleic acid that is less than 50 nucleotides long. This initial definition is updated today to include all synthesized nucleic acids at all lengths [123]. The composition of nucleic

acids and their role as genetic information carriers was known in the mid-forties last century. However, the molecular biology era was initiated first when Watson and Crick provided description of DNA double helix structure in earlier fifties [124]. In other words, it became more intriguing to decipher the relationship between chemistry and biological outcome. Hence, scientists attempted to synthetically replicate and exploit these macromolecules. Advances in technology but also supporting legislation aided these efforts that led to the birth of synthesized oligonucleotides [123]. First synthesized oligonucleotides came to the scene in 1950s by Khorana's group and others. They used phosphonate-H chemistry which was eventually incorporated in solid phase synthesis. Later on, di- and tri-phosphite chemistries were developed followed by the phosphoramidite chemistry of oligonucleotides introduced by Caruthers et. al in 1980s. Currently, the first-choice synthesis method is automated solid-phase synthesis (SPS) in which each nucleotide is incorporated as phosphoramidite chemistry [125].More about SPS will be discussed in the section (6.4).

Oligonucleotides are applied in therapeutics as chemically modified ASOs that target cellular RNA [126]. Moreover, oligonucleotides serve as biorecognition molecules that exhibit structural alteration upon interaction with a target biomolecule. This enabled exploiting oligonucleotides in diagnosis as smart activatable probes adapted in many colorimetry, electro chemistry and fluorescence-based biosensors [127, 128], and imaging modalities [129, 130]. In principle, each oligonucleotide is conjugated with a reporter at one end and a quencher at the other end. Upon activation the emitted signal of the reporter is increased [129, 131]. Target molecules include mRNA, hybridization with which results in conformational change and signal increase of a smart oligonucleotide probe. Such oligos are known as molecular beacons [129]. Importantly, oligonucleotides are degradable by nucleases [1]. Hence, a cleaving nuclease activity is an optimal target of such nucleic acid probes, provided that substrate oligos are sufficiently sensitive and specific to report on such an enzymatic activity [132].

Specifically, biosensors based on nuclease-activatable oligonucleotides have been used to detect various conditions [133] such as infections [70, 134, 135] and cancer [67-69].

Resistance to nucleolytic degradation can be tuned by implementing chemical modifications [136]. Chemical modifications can be incorporated in the sugar motifs, phosphate backbone and nucleobases of oligos. This enables modulation of cellular uptake, protein-binding and most importantly the stability against nucleases [136]. As such, a specific substrate to a target disease-nuclease activity could be designed. Sugar ring modification at the 2' with a methyl group (2'OMe), a fluor group (2'F), or conformationally locked nucleic acids (LNA) confer stability against nucleases [137]. For examples, incorporation of a methyl group in the 2' (2'OMe modification) hinders inherently the nucleophilicity of the oxygen, but also hinders sterically cutting by nucleases [91].

The oligonucleotides described in this thesis are the molecular detection tools that target nuclease activity. In other words, we design oligonucleotides to function as specific substrates to cancer associated nuclease activity that is the biomarker. They are synthesized by solid phase and serve as substrates for disease-associated nucleases. Nuclease activity is assayed for healthy vs. disease against a pool or a library of nucleic acid probes. The library comprises natural or chemically modified nucleic acids with 2'OMe, 2'F, or LNA as shown in the **Figure 4**. Best performing probes that are more degraded by disease-associated nuclease activity compared with healthy control are identified. Next generations could be designed based on the sequences of the identified best performing probes. Screening and probe design continue in rounds to enhance sensitivity and specificity of the oligo to generate, optimally, oligonucleotide(s) only degraded by disease but not healthy nucleases. The stringency can be modulated for instance by changing the type of chemical modification but also the type and location of the oligonucleotide across a sequence. The probes are FRET- probes flanked with a fluorophore and a quencher, at 5'- and 3'-end, respectively. A compatible plate reader detects the emitted signal upon cleavage by nucleases allowing to quantify nuclease activity.

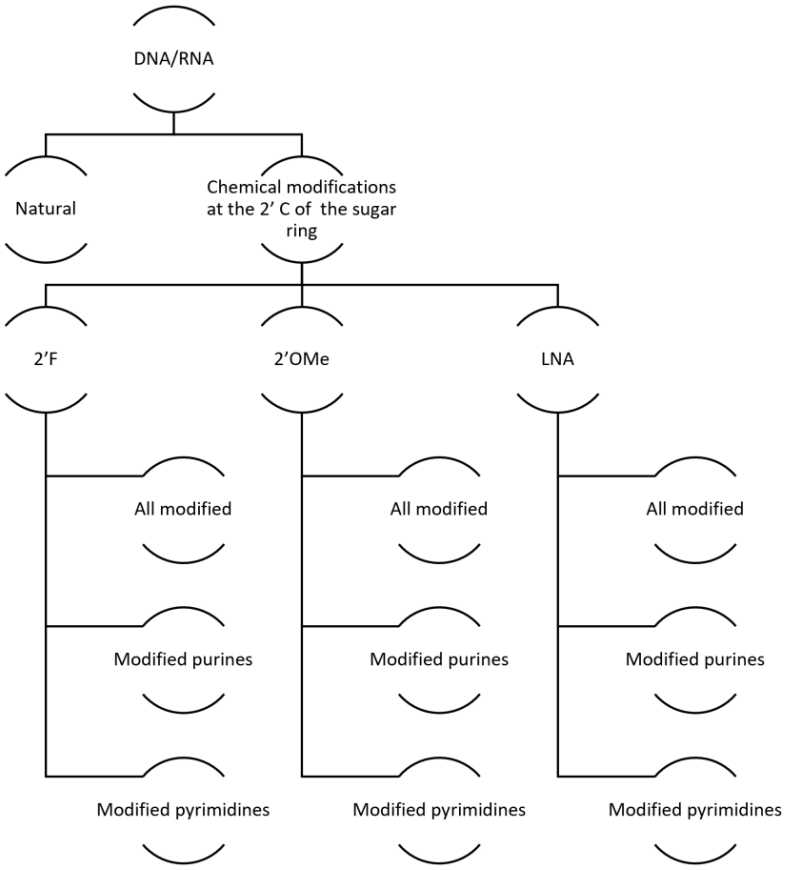

Figure 4. A classification scheme of the nucleic acid probes utilized in this thesis. Sequences of natural DNA and RNA, besides chemically modified probes on the carbon 2' of the sugar were utilized in screening for nuclease activity. The chemical modifications included 2'F, 2'OMe besides locked nucleic acids (LNA).

Chapter 3

3. Applications of Nucleases

3.1 Infections

Rapid and reliable detection of infectious pathogens is essential for proper treatment. Bacterial nucleases have been exploited in bacterial infection diagnosis with several means [2]. Detection of *Nuc* gene, that encodes thermostable nucleases, by PCR is utilized to diagnose *S. aureus* infections in food and clinical samples [138]. More recently bacteria associated nuclease activity was utilized as biomarker [131, 139, 140]. We and others have recently reported on the detection of *S. aureus in vitro* and *in vivo*, using micrococcal nuclease (MN) activity as a biomarker. As a substrate, a nucleic acid probe that sensitively and specifically is degraded by MN was utilized [139]. The sensitivity, specificity and efficiency in human serum and plasma of this probe in detection of *S. aureus* clinical isolates and laboratory strains was validated by other researchers. And it was shown that the sensitivity was enhanced by introducing a subtle change to the nucleotides order in the sequence. Thus, it was confirmed that utilizing nuclease activity towards activatable nucleic acid probes is a significant novel method for accurate and rapid diagnosis of bacterial infections [131]. As such, nuclease activity was also used for Salmonella detection [140]. Additionally, researchers developed a rapid assay for UTI diagnosis based on *E. coli.* endonucleaseI activity as a biomarker. The substrate is a nucleic acid probe that is specifically degraded by this bacterial nuclease in urine samples [70]. Rapid test that identifies microorganisms based on nuclease activity in real time was also suggested. A panel of fluorescent probes served as substrates in this method [141]. Nuclease activity was also recruited as a biomarker to track presence of *S. aureus, S. Typhimurium* and *S. Enteritidis*. By using plasmon assisted colorimetric assay, food contamination by bacteria was confirmed in two hours [142]. This body of research reflects on the diagnostic applications offered by the wide diversity of bacterial nucleases. Recently, a review was published detailing the utility of extracellular bacterial nucleases as diagnostic biomarkers for bacteria detection [37].

3.2 Autoimmune Diseases

Autoimmune diseases include a panel of maladies the basis of which is disturbance in immune tolerance. Immune system, in these diseases, attacks self-antigens in a systemic manner or limited to some organs [143].The list includes but is not limited to SLE, inflammatory bowel diseases (IBD) essentially Chron's disease, type 1 diabetes (IDDM), hepatitis and rheumatoid arthritis (RA), multiple sclerosis, primary biliary cirrhosis, myasthenia gravis, autoimmune thyroiditis, bullous pemphigoid, celiac disease and Sjögren's syndrome [26, 144]. Deficiency of extracellular nucleases, that digest circulating cell free nucleic acids, was proved to contribute to the pathogenesis of autoimmune diseases [26].

When undegraded, extracellular nucleic acids are both inflammatory and autoimmune triggering elements. Auto antibodies bind to self-DNA and form DNA-Anti-DNA complex, which is one of the features of autoimmune diseases such as SLE. These complex compounds trigger downstream inflammatory pathways and lead to damaging tissues they harbor [15]. Hence, alteration in DNase expression and/or activity was demonstrated to be correlated with and suggested to be potential diagnostic biomarker for a panel of autoimmune diseases [26]. Indeed, downregulated DNase activity was suggested as a biomarker but also a pathological driver of SLE [25].

DNase1, DNase1L1 and DNase1L3 are extracellular DNases that have major role in regulating the homeostasis endogenous cfDNA. Hence, it was hypothesized that any abrogation in the expression or enzymatic activity of these nucleases contributes to development of autoimmune diseases. The effect of DNase1 in this regard was shown to be redundant, while DNase1L3 disturbance is essential driving factor in autoimmune diseases development [26].

For example, aberration in DNase1 family is associated with SLE. Mutated *DNase1L3* that lacks DNase activity was found to be present in individuals with a familial form SLE [38, 39]. Sporadic SLE, on the other hand is associated with hypomorphic mutations of the gene [39]. Mutated variant of secreted DNase1L3 with abolished endonuclease activity was associated with hypocomplementemic urticarial vasculitis syndrome (HUVS) [41].

3.3 Obesity

Obesity, metabolic syndrome, and inflammation are closely related. In that, chronic inflammation in the metabolic adipose tissue is an integral part of obesity mainly through activation of immune cells targeting adipose tissue. This metabolic inflammatory state together with the endoplasmic reticulum (ER) stress are drivers in obesity development. ER stress increase adipose tissue macrophages activation, which in turn regulates metabolic inflammation. The macrophage inositol-requiring transmembrane kinase endoribonuclease-1α (IRE1α) pathway contributes to metabolic syndrome and obesity. And the role of IRE1α in promoting obesity based on overnutrition was demonstrated in a mouse model. Myeloid-specific IRE1α-knockout mice did not develop metabolic syndrome or diabetes. These findings suggest IRE1α as a therapeutic target to treat metabolic syndrome and obesity [42]. Moreover, a study conducted on mouse model found that endonucleaseG knockout in mice does not correlate with changes in mitochondrial oxidative metabolism, while it is correlated with reduced fat deposition and improved glucose tolerance [43].

3.4 Cataracts

Degradation of the nuclei is an important milestone in differentiation of lens fibers and suggested to be essential for lens clarity. DNase2β (DLAD) that is uniquely expressed in lens cells, both in mice and human, catalyzes degradation of nuclear DNA under the differentiation process [44]. *DNase2β* knock out (KO) mice developed cataract due to accumulation of undegraded DNA in the lens cells, which warrants for a further research on the possible deficiency in *DNase2β* gene in cataract patients[44]. In another study, a decreased expression of DNase2β mRNA followed by retaining non-degraded nucleosomal DNA were found in cataractous rat animal model [45]. In zebrafish, DNase1l1l is the nuclease that is exclusively expressed in lens. DNase1l1l KO zebrafish model generated using CRISPR CAS technology developed cataract. Abolished denucleation (nucleus clearance) was caused by the gene deletion in the animal model [46]. Altogether, data suggest the importance of DNases in the cataract pathogenesis, opening the door for applications in diagnosis but also therapeutic intervention.

3.5 Other Conditions

Given the high diversity in biological roles of nucleases and their capability nucleic acid degradation, nucleases can be harnessed as biomolecules to monitor or diagnose a wide array of diseases including the psychological disorders. In addition to the diseases discussed above, nucleases are involved or have been interrogated for their potential diagnostic value in a panel of conditions including asthma, parakeratosis, and xeroderma pigmentosa. In principle, any condition in which an alteration in nucleases' expression and or activity is present, is apotential a field for nuclease and nucleic acid probes diagnostic applications. For example, DNase1 activity in serum was suggested as a diagnostic biomarker for acute myocardial infarction [47] [48]. DNase1 is also involved in apoptosis [15], and DNase1 activity in serum was suggested as transient myocardial ischemia biomarker [49]. The DNase1 activity was measured by single radial enzyme diffusion method [101]. Additionally, nuclease deficiency in tear was shown to be associated with dry eye disease, resulting in insufficient clearance of ecDNA and NETs that accumulate and cause inflammation [145]. In a recent study on schizophrenia, it was demonstrated that the nuclease activity of DNase1gG abzymes recovered from serum of patients was higher compared with healthy [51]. Moreover, many studies have suggested a panel of nucleases as potential diagnostic biomarkers in several types of cancer. The application of nucleases as promising cancer diagnostic biomarkers is discussed in the chapter 4.

Chapter 4

4. Cancer Nucleases

4.1 Nucleases as Potential Biomarkers in Cancer Diagnosis

Cancer is among the leading causes of death worldwide [146]. It is highly heterogenous disease on inter-patient, inter-tumor and intra-tumor levels [147]. Early detection is a cornerstone for a good treatment management and survival [146]. Biomarkers are aimed to enable early detection of the disease and some clinically available biomarkers have a proved role in decreasing mortality due to the early diagnosis, where treatment options are effective [146]. Biomarker is defined as any measurable characteristic that indicates normal or pathogenic process, or response to intervention including a therapeutic intervention. Biomarkers could be molecular, physiologic, histologic, or radiographic [148]. In this regard, many nucleases have been the topic of research that indicated their potential utility as diagnostic biomarkers for various cancer types such as prostate cancer [54], gynecologic cancers [149, 150], breast [151, 152], head and neck squamous cell carcinoma (HNSCC) [17], colorectal [153] and others as reviewed in paper I [36].

Cancer develops due to mutation in oncogenes or tumor suppressors. Mutation or aberration in chromosome rearrangement results in dysregulated genes. A nuclease or nucleases could be any of the mentioned proteins, and a dysregulation in such nuclease could be correlated with cancer [119]. RNase5 can trigger oncogenesis and it is overexpressed in several cancer types [107]. Non-metastatic protein 23 H1 (NM23-H1) was suggested as a tumor suppressor and is downregulated in cancer [154, 155]. RNaseT2 is another example on downregulated tumor suppressor nuclease in cancer [118] The nucleolytic activity is a common feature of nucleases. However, they are involved in diverse biological pathways and functions [4, 25, 64]. Therefore, it is logical that differential status between normal and cancer varies for each nuclease and cancer type.

Some nucleases are a part of DNA repair machinery, such as the nucleases APE1, XPF/XPG, FEN1 and MRN complex [13]. Downregulated DNA repair is associated with carcinogenesis on one hand, and upregulated DNA repair is correlated with chemotherapy or radiotherapy resistance on another hand. Besides, germline mutations in DNA repair genes contribute to accumulated DNA mutations associated with, among others, cancer without a repair response. That explains the correlation between such mutations and cancer [13]. FEN1 is overexpressed in breast, colorectal, ovarian, gastric, lung and kidney cancer [13, 152, 156, 157]. APE1 is upregulated in ovarian, lung, prostate, head and neck cancer and melanoma [13]. Overexpression was observed in breast cancer [158].However, it is downregulated in ER-positive breast cancer [13]. High expression of XPF is found in lung, cervical and ovarian cancer. High expression of XPG is associated with ovarian and gastric cancer [13]. Low expression of MRN complex is associated with breast colon gastric and bladder cancer [13, 159].

In addition to the afore mentioned nucleases, other ones are upregulated in association with cancer. SND1is upregulated in hepatocellular, breast, prostate and colon cancer [160-164]. In a recent study, RNase4 expression was found to be upregulated in tumor tissue and plasma from prostate cancer patients. Plasma concentration was positively correlated with stage and grade [54]. Moreover, genetic variation of a nuclease could predict cancer risk. For example, specific SNPs of *RNase3* are correlated with a high risk for colon cancer [165]. DNase1 phenotype 2 is highly frequent in gastric and colorectal cancer [166, 167]. RNaseL gene polymorphism is associated with prostate cancer [61], and a specific SNP is correlated with increased risk of head and neck, breast and uterine cancer [168].

Most of these and other nucleases were discussed in detail in paper I that provides a literature overview of nucleases reported as potential cancer diagnostic biomarkers. RNase5 and RNaseT2 were not included in the paper and are therefore selected to be discussed in this section.

RNase5, along with the other hcRNases, contributes to the host defense against pathogens as it exerts an antimicrobial effect. RNase5 is also known as angiogenin (ANG) because it promotes formation of blood vessels (angiogenesis), a key process for tumor growth and invasion [149,

150]. Although the catalytic activity is weak, it is required for angiogenesis [112]. Increased serum level of RNase5 was correlated with pancreatic cancer aggressiveness [55]. And RNase5 upregulation was correlated with HNSCC [112]. Moreover, expression is elevated under hypoxic conditions in specimen and human cell lines of oral squamous cell carcinoma [56]. In gastric cancer, serum concentration of RNase5, measured with ELISA, was remarkably higher than that in serum retrieved from non-neoplastic patients or healthy donors. Moreover, a positive correlation between elevated serum concentration and cancer progression was found [57]. RNase5 protein expression in tumor samples was positively correlated with both resistance for radiotherapy and recurrence in patients with nasopharyngeal carcinoma. This indicated a predictive and prognostic role of RNase5 [169]. Immunohistochemistry (IHC) analysis visualized a significantly increased expression of RNase5 in prostatic adenocarcinoma and its precursor high-grade prostatic intraepithelial neoplasia compared with adjacent benign prostate tissue [58].

In breast cancer, studies demonstrated an elevated RNase5 expression in breast cancer tumors compared with healthy tissues [59, 60]. Higher concentration was also observed in sera from breast cancer patients compared with healthy counterparts [59]. Serum levels did not show any prognostic value in contrast to tissue levels that were positively correlated with good prognosis in terms of overall and disease-free survival (DFS) [59]. However, same conclusion was not reached by another study [60]. RNase5 was indicated to contribute to breast cancer progression. Due to the positive correlation between the protein expression in tumors with the estrogen receptor (ER) status, it was suggested to be a target for tamoxifen [60]. Moreover,RNase5 is found to be a ligand that directly binds to epidermal growth factor receptor (EGFR) causing the phosphorylation of the latter and its downstream pathways activation, promoting a malignant transformation. RNase5 exert antimicrobial activity and can promote oncogenic transformation independently from its nucleolytic activity [107].

Upregulated serum RNase5 was also found in patents with invasive cervical cancer compared with cervical intraepithelial neoplasia (CIN) and healthy individuals, suggesting a correlation between the RNase5 upregulation and invasiveness [170]. This finding is in line with another

study that demonstrated a positive correlation between serum levels and tumor stage [149]. An elevated RNase5 mRNA expression was found in colon and gastric adenocarcinoma [111]. Higher protein and mRNA expression in colorectal cancer tissues was demonstrated by IHC and in situ hybridization, respectively, compared with adjacent healthy tissues. The serum levels were coherent with the protein levels in tissue and were negatively correlated with disease-free or disease-specific survival [171]. RNase5 is upregulated in many other cancers including colon [111], astrocytoma [172], bladder [173], renal [174], myeloma [175], leukemia [176], osteosarcoma [177] and melanoma [178].

Human RNaseT2 is suggested as a tumor suppressor as it has an inhibitory effect on ovarian cancer [117, 118]. Interestingly, the ribonucleolytic activity is not essential for the cancer inhibition. Noteworthy is that the tumor-suppressive activity in ovarian cancer is exerted *in vivo*, demonstrated with xenograft models, but not *in vitro*. The gene expression is downregulated in melanoma cell lines, ovarian cancer cell lines and tumors. A decreased expression was also observed in glioblastoma and lymphoma [118]. Additionally, deletions in the chromosomal region in which *RNaseT2* is located, is correlated with several cancers [118] including breast, colorectal , stomach, ovary, uterus, liver, kidney, melanoma and parathyroid gland cancers as well as a panel of hematologic cancers [179]. The control of tumorigenicity in vivo was also reported in human colon cancer. In particular, treatment with recombinant RNaseT2 reduced the clonogenicity of HT-29 cells *in vitro*. An antiangiogenic activity was demonstrated *in vitro*. Besides, both tumor-suppressive and antiangiogenic effects were shown in animal models with human colon cancer xenografts [179]. An antagonizing effect on melanoma cells tumorigenesis was also demonstrated [180]. The inhibitory activities on tumorigenesis and angiogenesis mediated by its intracellular and extracellular functions, combined with its safety as a human RNase proposed a therapeutic potential for the recombinant RNaseT2 [181].

4.2 Nuclease Activity as a Cancer Biomarker

As discussed in the previous section, research has provided evidence that multiple nucleases display an alteration in expression and/or activity in cancer. In several cancer types, nucleases are upregulated. Differential

status of nucleases between healthy and cancer has been extensively investigated for its diagnostic potential. And determining this difference relied heavily on techniques that analyze protein expression, gene expression or mRNA and mutational analysis. The enzymatic power of nucleases has been less exploited in this regard.
Decreased enzymatic activity of RNaseL variant was observed in prostate cancer using rRNA substrate [182]. Increased global ribonuclease activity in the serum was correlated with pancreatic cancer. To assay nuclease activity, t-RNA (T) from *E. coli* and polycytidylic acid (poly-C) were used as substrates, and bovine RNaseA was used as a reference enzyme [183].

The flexible synthesis of natural and chemically modified oligonucleotides has facilitated exploiting these molecules in biosensor applications aimed for diagnosis [127], among others as substrates for nuclease activity [135, 184]. In HNSCC, decreased catalytic activity towards dsDNA of the R156L variant of TREX2 was reported. The nucleolytic activity was assayed against 30-mer dsDNA oligonucleotides [17]. Moreover, Hernandez et al described how SKBR3 breast cancer cells were discriminated from healthy fibroblasts based on cell membrane nuclease activity profile towards designed oligonucleotide substrates. Higher degradation of 2'-O-methyl modified FRET-probes was observed for SKBR3 cells compared with other cancer and healthy cells, providing an evidence on nuclease activity as a promising biomarker for cancer detection [67]. Furthermore, circulating cancer cells in blood samples from breast cancer patients were detected based on cancer cells associated nuclease activity and nucleic acid probes as substrates [68]. In another study, cell membrane bound nuclease activity was investigated using an inhouse developed surface-tethered nuclease sensor, a different biosensor approach from the previously mentioned solution-based ones. The study demonstrated that cancer cells were associated with higher nuclease activity compared with healthy cells, observing both spatial and temporal dynamics of nuclease activity. Additionally, single breast cancer cell was detected among thousands co-seeded adherent healthy cells or millions mixed blood cells, using cancer associated nuclease activity as detection means [185]. Collectively, these studies are suggesting the diagnostic power of nuclease activity as a biomarker. Realizing the limitations of the current cancer biomarker landscape such as low bodily concentration and heterogeneity in time of occurrence and abundance in individuals [186]

prompt further research in the avenue of nuclease activity as a potential biomarker in cancer detection.

From analytical point of view, nuclease activity assay is a rapid diagnostic method with turnover time of minutes-hours [67, 139]. No amplification step is required due to the inherent dynamic nature of nuclease activity, in that each nuclease degrades many reporter oligonucleotides. Therefore, the detection limit is at picomole or femtomole level. Combined with all the virtues of the nucleic acid probes as biorecognition tools, nuclease activity detection provides an added benefit to cancer diagnosis landscape

Chapter 5

5. Breast Cancer

5.1 Breast Anatomy

The breast is the paired mammary glands, located over the pectoral muscle on the superior region of the anterior thoracic wall. In female, mammary gland produces and secrets milk under influence of hormones, such as prolactin. The three main components of the breast in adult women are glandular, connective and adipose tissues. The glandular part is composed of 15-20 lobes. The lobes are subdivided into lobules that consist of glandular acini that produce milk. Lobules are connected with branched ducts functioning as transport system of milk to the nipple. Beneath the areola, ducts delate and form milk-storing sinuses [187, 188]. The lobes are surrounded by adipose tissue and supported by fibrous connective tissue that together are the stromal element. Stroma is the major component of the non-lactating breast. The breast is layered by thin skin the central region of which is called nipple that exhibits high pigmentation [187]. The **Figure 5** depicts a simplified structure of the female breast.

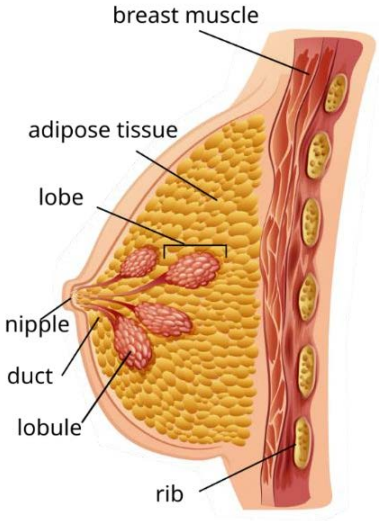

Figure 5. A simplified structure of the female breast.

5.2 Hormones and Breast Development

Breast growth starts during the embryonic life, and undergoes major structural and functional changes during puberty, pregnancy, lactation and menopause. The breast development in female is governed by ovarian hormones and associated with age milestones. The mature development is estimated to be reached at the age of 20. However, a complete development occurs with pregnancy and following lactation. During these events both function and volume of the glandular element are drastically impacted. Structural changes occur regularly with each menstrual cycle as well. By the age of 40, atrophic changes debut even prior to the actual menopause [187].

The breast consists of primitive ductal structure at birth that develops slowly into a more canalized and branched one under prepuberty. Breast development ends at this level in male, while it proceeds in female from puberty to adulthood. The pituitary gland hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH), stimulate maturation of ovarian cells and secretion of estrogens under puberty, which impact breast development. The response to hormones is manifested by changed shape, increased size and lobules formation. The latter is the result of both lobules development and differentiation [188, 189].

Type 1 lobules are the least developed and formed about 1-2 years following menarche. A lobule is made up of roughly 11 alveolar buds [188, 189]. Altered levels of estrogen and progesterone under each menstrual cycle mediates development into more differentiated types 2 and 3 that contain greater number but smaller sized alveoli. All three types could be present in the adult female breast. However, type 1 is the dominant in nulliparous women. During full-term pregnancy, lobules evolve to type 4 that is the most developed and differentiated. Lobules are also subjected to epigenetic remodeling during pregnancy, that is suggested to provide a long-lasting protective effect against breast cancer [189]. The structural changes of lobules sustain under lactation. Lobular involution, or regression, from type 4 to 3 supervene in post-lactational breast [189]. After menopause, lobules undergo regression into type1 both in parous and nulliparous women, affected by ceased secretion of ovarian estradiol and progesterone. The glandular compartment is substituted with fatty tissue [188]. Even connective fibers and stromal

cells decrease in number [187]. The difference is, however, the epigenetic changes under pregnancy that are conserved on long term of around 30-40 years [190].

5.3 Breast Cancer: Definition, Pathology and Diagnosis

Breast cancer arise from the terminal duct of the lobular units of the collection duct [66]. Female breast cancer is the most commonly diagnosed cancer type over the world [191]. In women, breast cancer accounts for 30% of cancer cases. The incidence ranges between 27 per 100000 in Africa and East Asia, and 97 per 100000 in North America. The decline in mortality is continuing however not evenly across the world [192]. About 80% of diagnosed population is over 50 years old. The survival varies depending on the molecular subtype and the stage [193]. Breast cancer is curable in 70-80% of the cases at early stage [66]. Early detection and improved treatment are the main factors in the decreased death rates that are estimated to further decrease by increasing the access to prevention, detection and treatment of high quality [192].

Breast cancer pathogenesis can not be described as straight forward due to many implications at the cell of origin, molecular, genetic and morphological levels. The malignancy may arise due to accumulated mutations and epigenetic changes that favors the clone with "fittest" cells to survive, or progress only driven by precursor stem cells. Stem cells could also evolve in a clonal manner. Morphologically, lesions and genetic changes from the gland occur. At the molecular level, breast cancer evolves in a low grade and proliferation pathway, or in a high grade and proliferation fashion. These pathways are defined based on ER expression, tumor grade and proliferative index and categorized in molecular subtypes. Besides, additional aspects of pathogenesis have been understood after identifying breast cancer susceptibility genes [66].

Mammography is an X-ray imaging modality that is implemented for detection of early signs of breast cancer or other diseases in breast [194]. Screening with mammography is abundantly available. Therefore, most of the breast cancer cases are diagnosed upon screening. A smaller cohort, however, is diagnosed with one or more of the following clinical features: a palpable bump in the breast or in the axillary area, breast skin erythema

or thickening, nipple inversion or discharges from nipple, and localized pain [66, 195]. The diagnosis is based on combining clinical examination with mammography and/or ultrasonography imaging and needle biopsy [66].

The aim with screening is to detect a disease in a specific population at an early stage at which an effective treatment is available [66]. Mammography is the standard screening method. On one hand, mammography screening is one of the factors contributing to decreased mortality. On the other hand, overdiagnosis and overtreatment issues remain present in this method [196]. Therefore, reliable diagnostic biomarkers for improved diagnosis are still required [53, 197]. MRI is used as a supplementary imaging method for screening women with increased risk for breast cancer. Evidence on increased 10-years survival and increased early-stage detection by MRI supplementary screening has been reported [196]. Based on evidence, breast MRI as adjunct screening method to mammography is recommended to several groups including but not limited to: breast cancer gene (*BRCA*) mutation, individuals not tested yet and first-degree relative to a *BRCA* mutation carrier, persons with a lifetime risk (LTR) of 20-25% or higher and persons that received recommendation of MRI screening based on experts' opinion [198].

As mentioned before, early detection of breast cancer relies heavily on screening with mammography, although there is a known risk of false positive results applying the method. No early detection biomarkers are currently available in the clinic despite the many attempts to characterize such. The biomarkers related to breast cancer are mainly useful for prognosis, predicting the response to therapy and monitoring the treatment response after initiating the therapy [197].

5.4 Heterogeneity of Breast Cancer

Breast cancer is highly heterogenous in contrast to the initial common idea of being homogenous. Diverse histological types, histological grades and molecular subgroups are present and refer to prognostic and predictive behavior of tumors [66, 199]. The most common histological subtypes are ductal carcinoma followed by lobular carcinoma that are both invasive [66, 192], and their preinvasive counterparts ductal

carcinoma *in situ* and lobular carcinoma *in situ* [66]. Ductal carcinoma is also known as no special type (NST) and constitutes 70-75% of breast cancer tumors [66].

Histological grade of a breast cancer tumor relies on the degree of differentiation of the tumor tissue based on a semi-quantitative assessment of morphological properties. Nottingham grading system (NGS) is applied in clinic, and it distinguishes 3 morphological characteristics: mitotic count, degree of tubule or gland formation, and nuclear pleomorphism. Each tumor is assigned a score for each characteristic, and the accumulative final score indicates the tumor grade (1,2,3). The lowest grade tumors (grade1) are more differentiated and highly homologous with normal breast tissue, with high tubular formation and low nuclear pleomorphism and mitotic count. This grading system has an established prognostic value and is still implemented in the clinic, but also integrated in algorithms to guide treatment decision [199].

Moreover, molecular classification was initiated by by *Perou et al* who aimed to classify tumors into categories with distinctive prognosis based on gene expression profiling (PAM50) [200]. This study classified breast cancer into four molecular subtypes luminal A and B that express ER, human epidermal growth factor receptor 2 (HER2)-enriched without ER expression), and basal- like (without ER, PR or HER2 expression). In addition, a normal like subtype was described. This subtype has no clinical relevance as it contains low number of tumoral cells in a majorly normal tissue **(Table1)** [66, 200]. A downsized yet relevant list of genes was proposed later on to provide less costly subtyping [201].
In clinic, surrogate intrinsic subtyping is adapted based on histological characteristics and the protein expression of ER, PR, HER2 and the proliferation marker Ki67, as indicated by a routine IHC staining [66]. These biomarkers are crucial to determine prognosis, stratification of treatment and prediction of treatment response [202]. When either ER or PR is expressed in at least 1% of the tumor cells, a tumor is considered hormone receptor (HR)+. HER2 is expressed in 20% of breast cancer tumors which are known as HER2+. When expression of ER, PR and HER2 is lacked tumors are known as triple-negative breast cancer (TNBC) [66].

Table1. The molecular subtypes of breast cancer, correlation with tumor grades, systemic treatment options, and other relevant characteristics.

Molecular subtype	Receptors' expression	Histological Grade	Histological type and other characteristics	prognosis	Systemic treatment
Luminal A-like	Strong ER+ and PR+, HER2-	low grade	NST, low Ki67 index, low proliferation	good	endocrine therapy, chemotherapy (if required)
Luminal B-like Her2-	ER+ (lower ER and PR than luminal A like), HER2-	higher grade	NST micropapillary and lobular pleiomorphic histotype, high Ki67 index	intermediate	endocrine therapy, chemotherapy (if required)
Luminal B-like Her2+	ER+ (lower ER and PR than luminal A like), HER2+	higher grade	NST and pleiomorphic, high Ki67 index	intermediate	endocrine therapy, HER2-targeting therapy, chemotherapy (if required)
Her2-enriched (non-luminal)	ER-, PR-, HER2+	high grade	high Ki67 index, NST	intermediate	responds to HER2 targeting therapy, chemotherapy (if required)
Triple negative	ER-, PR-, HER2-	high grade	high Ki67 index, NST or special histological type	Poor except for some special types	only chemotherapy

Furthermore, pathological stage and lymph node status are assessed upon diagnosis. TNM staging system is often applied to evaluate the extent of tumor (T) in the primary site, involvement of regional lymph nodes (N) and distant metastatic (M) sites. Five stages (0-IV) are generated by this system that is used in pathology report and treatment decision [66]. TNM staging does not take into account the molecular subtypes and is considered to not be enough to guide treatment decision [203]. The recent version of TNM staging takes into account the biological features of tumors [193].

5.5 Breast Cancer Risk Factors

A significant breast cancer risk factor is genetic predisposition or family history from maternal and paternal sides. In average, a family history is present in 10% of average breast cancer cases. Germline mutations in *BRCA1* and *BRCA2* increase the risk of an individual into 70% [192]. Another considerable factor is female gender. Breast cancer is significantly less common in males. Long hormonal exposure, such as early menarche or late menopause, is associated with increased risk [193]. Even medication with hormone replacement therapy and hormonal oral contraceptives is associated with increased risk that is positively correlated with the duration of use [193, 204].
Lack of breastfeeding and nulliparity are risk factors. While early pregnancy, especially in the early twenties, has a long-lasting protective effect. Among the possible explanations are decreased number of stem cells in the breast microenvironment, altered response to hormones, genetic changes in favor of increased differentiation and decreased proliferation [66, 193]. Other risk factors are older age and type 2 diabetes [192], higher breast density, and a personal history of breast cancer or benign alteration in the breast tissues. Risk is also correlated with race or ethnicity. Caucasian non-Hispanic women are the group with highest prevalence. Mortality, however, is higher in African women which is the group with the lowest survival rate [193].

The risk factors mentioned above are not possible to modify, others are modifiable. For instance, both active and passive smoking is associated with increased risk due to the accumulation of smoking carcinogens in breast tissues and thereby increased probability of mutations in among others *p53*. Women smoking before a full-term pregnancy are imposed to

additional risk. Medication with some antidepressants such as selective serotonin reuptake inhibitors, paroxetine and tricyclic antidepressants is correlated with elevated risk. Other drugs are also correlated with increased risk such as statins and antihypertensive medicines, for example calcium channel blockers and angiotensin II-converting enzyme inhibitors. Exposure to artificial light at night is another avoidable risk factor, a possible contributing factor to which is disrupted melatonin rhythm as a main contributor [193].

Moreover, sedentary lifestyle, high alcohol intake, and fat-rich and fiber-poor diet are all associated with higher risk [192]. Obesity in post-menopausal women is correlated with increased risk, and obesity at any menstrual status is associated with poor outcomes. Physical activity has a preventive effect.
Lack of D-vitamin is correlated with higher risk, although further research is demanded to draw credible data. Intake of ultra-processed food increases the risk, while consumption of turmeric-derived curcuminoids and specific ingredients in green tea is linked with cancer preventive effect, according to some *in vitro* and *in vivo* studies [193]. Preventive factors also include treatment with non-steroidal anti-inflammatory drugs (NSAIDs) during pre-menopausal life. The latter is thought to decrease the risk through lowering synthesis of prostaglandin, which is in turn involved in many inflammatory pathways [205].

5.6 Principles of Breast Cancer Treatment

Treatment strategy includes a combination of locoregional and systemic therapy, and treatment decision is based on the stage and molecular subtypes [193, 195]. The aim of therapy in early stage nonmetastatic breast cancer is tumor eradication from breast and regional lymph nodes in addition to prevention from metastatic recurrence [193, 195]. At early stage nonmetastatic breast cancer, tumors are surgically excised as a locoregional management, and a postoperative radiation might be applied. If required, systemic therapy is given preoperatively (neoadjuvant) or postoperatively (adjuvant) or both approaches are adapted. Neoadjuvant therapy, although controversial, aims to reduce the tumor burden and assess prognosis, especially in HER2+ and TNBC tumors. Adjuvant therapy, on the other hand, aims at reducing recurrence risk and is given based on the surgical results and biomarkers [66, 193].

Systemic therapy includes endocrine therapy (tamoxifen, anastrozole, letrozole or exemestane) to all patients with ER+ or PR+ tumors, to inhibit ER. For patients with HER2+ tumors, also anti-HER2 monoclonal antibody is given (trastuzumab or pertuzumab). Chemotherapy can be given to all subtypes when required, and represent the only systemic treatment option to patients with TNBC [195]. Basically, same systemic treatment is applied in metastatic disease, also surgery and radiation are performed, to palliate and prolong life [193, 195]. Given the high heterogeneity of breast cancer, research towards individualized therapy strategies per tumor and patient is increasingly performed [193].

Chapter 6

6. Main Methods

6.1 Screening

At its very early existence, screening was termed searching for active molecules and was applied on extracts from plants or animals. Technology evolution in 1980s led to emergence of screening as a process in which activity of novel synthetic compounds on one or more targets is tested. The era of robotics and automation scaled up the process to high-throughput screening (HTS) in 1990s, mostly performed by pharmaceutical industry [206].
Today, up to hundreds of thousands of molecules are screened by HTS [206]. Systematic evolution of ligands by exponential enrichment (SELEX) and phage display are other screening technologies that identify aptamers [207] and peptides [208] with high affinity to target molecules, respectively . Screening for nuclease activity presented in this work (papers II , III and IV) aims to identify nucleic acid probes that target sensitively and specifically disease-associated nuclease activity. Probe libraries include 20-75 oligonucleotides.

6.2 FRET

FRET was established in 1948 [209], and ever since it has been efficiently used in bioimaging and molecular detection [210]. The photophysical principle behind FRET is comprehensively reviewed in [211]. Briefly, FRET is a nonradiative process that is based on energy transfer from a donor dye molecule upon excitation to a proximate acceptor dye molecule that is in its ground state. The donor accepts a photon upon excitation and jumps from the ground level (S_0) to the excited energy level (S_1). Subsequently, the energy is transferred to the acceptor, that in turn absorbs the energy and emits a signal when it returns to its ground state (**Figure 6**) [212]. Three conditions are required for FRET to be achieved. 1) A spectral overlap must be present between the emission of the donor and absorption of the acceptor. 2) The spatial proximity between the donor and accepter must be close enough, maximum 10nm. 3)The donor and the acceptor must be in a correct orientation to each other [211].

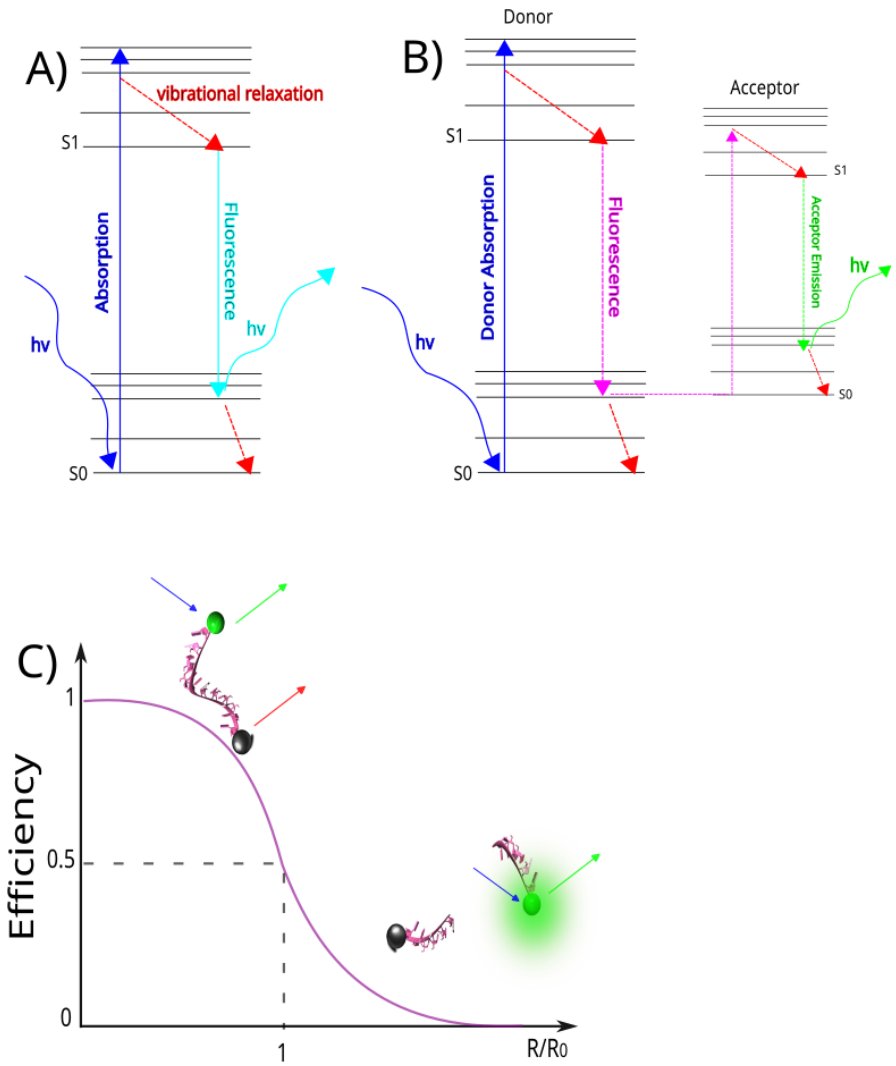

Figure 6. The basic principle of FRET in fluorescent probes. A) In Jablonski diagram, an electron rises to excited energy state upon absorption of a photon by a fluorophore. Due to instable excited state, a relaxation back to the ground state takes place within few nanoseconds after the excitement. A photon is emitted with a longer wavelength than the absorbed due to the energy quantum of this difference. B) In Jablonski diagram, the energy relaxation released by the donor is transmitted to the acceptor located in close proximity. As a result, an electron is excited in and a photon is emitted by the acceptor. C) The correlation between the efficiency of FRET and distance between fluorophore and quencher in FRET-based probes. R0 represents the Förster radius which is the distance where 50% of energy is transferred with FRET. (Inspired by the ref [214]).

FRET-based probes are among the most common ratiometric probes in studying molecular dynamics due to their multifaceted capability of sensing protein-protein interactions, enzymatic degradation, distance from cell membrane and intracellular signaling among others [210]. The nucleic acid probes utilized in the papers II, III and IV are labelled with FAM as fluorescent donor at 5', and TQ2 as a dark acceptor at the 3' termini, respectively. TQ2 has an optimal absorption maximum that matches FAM emission, rendering these dyes to a perfect donor-acceptor pair [213].

6.3 Cell Lines

Human cell lines are an important element in research that represent *in vitro* disease models such as breast cancer [214]. The first established human cell line was HeLa, that is derived from cervical cancer. George Gey was the scientist behind establishing this cell line in his lab in Baltimore over 70 years ago [215]. In 1958, BT-20 was established as the first breast cancer cell line [216]. About two decades later, several breast cancer cell lines were established [214]. Cell lines represent an infinite source that is highly homogenous, easy to handle and genetically modify on one hand [217]. On the other hand, cell lines could alter genotype or phenotype over long term use, which can be avoided by cryopreservation and using cells with only low passage number in experiments [217]. The ability of cell lines to fully reflect the heterogeneity of breast cancer and mimic physiological microenvironment still needs improvement [214]. In this thesis (paper III), 9 breast cancer cell lines were used: BT-474, MDA-MB-231, MDA-MB-461, SKBR3, BT-20, , MCF7, ZR-75-1 and T-47D. Additionally, BJ and CCD-18-Lu normal cell lines were used as healthy control.

6.4 Solid-Phase Synthesis of Oligonucleotides

Automated SPS is the most common technique to synthesize nucleic acids. The name refers to the use of solid support which is non-soluble material, commonly controlled pore glass (CPG) or polystyrene, to which oligos are attached in synthesis. In SPS, nucleotides in phosphoramidite form are linked into a growing sequence through cycles of 4 steps each. First, the protecting 4,4´-dimethoxytrityl (DMT) groups are removed

from the 5'end of nucleotides pre-attached to the solid surface. The process is known as detritylation. Next, the phosphoramidite nucleotides building blocks are activated with 5-(benzylthio)-1H-tetrazole and linked to the 5'-OH of nucleotide integrated in the solid support. The third step is capping the unreacted 5'-OH groups in the solid support attached nucleotides. This step is important for prevention of further sequence elongation with a single nucleotide deletion. In the last step, the unstable phosphite triester is oxidized into the stable phosphate by aquaeous iodine [125]. The oligonucleotides in this thesis (paper II, III, IV and V) are synthesized by standard SPS, and high-performance liquid chromatography (HPLC) was applied as post synthesis purification step. Probe synthesis and purification were performed by Biomers.net (Germany). Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) was used to confirm probe synthesis. FRET probes were fully synthesized by Biomers.net. TT-probe (paper V) was synthesized with amine group at 5'-end and azide group at 3'-end by the same manufacturer to be coupled with D-SPION and gadolinium complex at the 5'-end and the 3'-end, respectively.

6.5 Tissue Samples

Both freshly obtained and frozen human specimen has aided research in therapeutic and diagnostic areas. This clinically relevant material helps in elucidating pathways, evaluating therapeutics and identifying molecular targets. For example, HER2 was first identified in human tissues repository. Based on this observation, targeted therapy against HER2 was developed. Consequently, poor prognosis of HER2 expressing tumors was changed by this therapy into good [218].
Breast tissue samples in this thesis (paper III) were obtained from the Biobank of the Basque Country, Spain for the retrospective arm of the study. For the protrospective arm, tissues were received from patients upon surgery or surgical biopsy and assayed within 1 hour for nuclease activity.

6.6 Transmission Electron Microscopy

Transmission electron microscopy (TEM) is based on a similar principle to that of the light microscopy. However, electrons are used instead of light. Electrons wavelength is remarkably lower than visible light

wavelength, and therefore higher resolution is provided by TEM. Upon confronting a thin sample of <100nm, electrons are scattered. Contrast is the result of differently scattered electrons based on the surface they encounter. An image is generated through focusing and magnifying the scattered electrons. This technique enables acquiring images with atomic level resolution. TEM is one of the best methods for nanoparticles characterization [219], and is therefore utilized to characterize size and morphology of the activatable MRI probe (paper V).

6.7 Dynamic Light Scattering

Dynamic light scattering (DLS) is utilized to measure size and size distribution of particles at the submicron region. The technique is typical for characterization of particles after dispersing or dissolving in liquid. Detailed explanation of DLS and its applications is provided in [220]. In brief, the principle is measuring the fluctuation of intensity of light scattered by particles that are diffusing with a Brownian motion. Fluctuation is dependent on diffusion rate. Larger particles undergo slower motion, and hence less fluctuation in the intensity of scattered light is resulted in. Scattered light signals are captured and compared over the time, and equations are used to calculate particles' hydrodynamic size and size distribution. Hydrodynamic diameter of both the DMSA-coated superparamagnetic iron oxide nanoparticles (D-SPION) and activatable MRI probe (paper V) was assessed using DLS.

6.8 UV–Visible Spectroscopy

Ultraviolet-visible (UV-Vis) spectroscopy is utilized as a rapid, flexible and non-invasive method to characterize material in aqueous solution. This technique measures absorbance or transmittance of light with a working wavelength of 200-800nm. Information on absorption band, formation, concentration, electronic properties and stability of the measured species is obtained using UV-Vis spectroscopy [221]. The technique is ubiquitously applied in characterization of magnetic nanoparticles [222]. In this work (paper V), UV-Vis was utilized to measure the light absorbance of D-SPION, TT oligonucleotide probe, and the activatable MRI probe to confirm integration of the TT oligonucleotides in the MRI probe.

6.9 MRI, Contrast Agents and Relaxivity

MRI is an imaging modality that is based on the radiofrequency signals from protons upon exposure to a strong magnetic field [223]. MRI stems from nuclear magnetic resonance (NMR), a technique that measures magnetic characteristics of atomic nuclei. MRI measures the nuclear resonance of protons of water molecules in the body upon exposure to an external magnetic field [224]. It was in the early 1970s NMR was used in biomedical field, namely, to measure T1 and T2 relaxation times in rat tumors. The technique became more mature when magnetic field gradients were implemented, which lied the foundation for currently known MRI [225]. Today, MRI is ubiquitously utilized as an imaging modality in preclinical and clinical settings [223, 225]. MRI offers non-invasive imaging with high special resolution and excellent penetration that is preferably used for imaging soft tissues [223].

MRI sensitivity is inherently low due to the abundance of water in the body and thereby lack of contrast between soft tissues. To overcome this issue, contrast agents (CAs) are used. Two types of CA are utilized: T1- and T2- weighted. T1-weighted CAs are also known as positive CAs, and they shorten the longitudinal relaxation time T1of protons and generate a brighter image in T1-weighted MRI. T1 agents are usually paramagnetic such as Gd^{+3} complexes, that are the most common contrast agent applied in the clinic [225] [226]. T2-weighted CAs, on the other hand, are superparamagnetic such as SPION, they shorten the transverse relaxation time T2 of protons and generate a darker image in T2-weighted MRI [224]. However, it is not always that CAs solve the problem of sensitivity. Therefore, tissue specific CAs or activatable CA nanoparticles are increasingly implemented [226].

In this work (paper V), activatable MRI probes were synthesized based on TT-oligonucleotide that is specifically degraded by MN. From the 3'-end, TT-probe is coupled with D-SPION, and the 5'-end with dendron functionalized with several Gd^{+3} complexes as a contrast agent. A preclinical MRI with a 9.4T magnetic field was used to evaluate the imaging property MRI-probe *in vitro*, and T1 values were estimated.

Furthermore, the efficiency of CA is determined by relaxivity that is r_1 and r_2 for *T1* and *T2* CAs, respectively. Relaxivity refers to the extent to which a CA can impact the relaxation rate of water [226]. In this thesis (paper V) an NMR analyzer with magnetic field of 1.41 T (inversion time) was used to measure T1 and T2- relaxation times. Next, 1/T1 values

were plotted against different concentrations of the measured entities. The relaxivity r_1 was determined by calculating the slope of 1/T1 graph. This was applied to evaluate the "magnetic quenching" and the efficiency of the MRI-probe in detection of target bacteria. T1 and T2- relaxation times were measured for a concentration series of gadolinium and the activatable MRI probe, for the evaluation of "magnetic quenching" and for evaluating the detection efficiency of MRI-probe, respectively.

Chapter 7

7. Summary of the Papers

Paper I. Nucleases as Molecular Targets for Cancer Diagnosis

A literature review is presented in paper I to discuss nucleases frequently studied for their promising diagnostic utility in cancer. Nine nucleases have been discussed in addition to serum RNase activity. For each nuclease, an introduction about the catalytic activity and biological functions was provided. It was detailed in which cancer type a nuclease was investigated and which analytical techniques were utilized. Additionally, utilizing nuclease activity in cancer detection using nucleic acid probes as substrates was also highlighted by referring to our and others' work.

A plethora of studies are published, with the majority reporting on one nuclease of interest per cancer type. However, looking at the overall picture, several nucleases are altered in term of expression and/or activity in a cancer type. Additionally, a given nuclease is involved in more than one cancer type and sometimes with different alterations or subcellular abundance depending on the type. For instance, nuclear expression of APE1 is higher than the cytoplasmic in gastric cancer tissue cells. In prostate cancer, however, overexpression is present in both nuclei and cytoplasm. Even the correlation between the overexpression status and the outcome could be different from a cancer type to another for the same nuclease. For instance, the decreased expression of XPF/XPG is correlated with better treatment response and survival in many cancer types while an increased expression of ERCC1, that activates XPF through heterodimerization, was correlated with a better outcome in gastric cancer [227].

The analytical techniques used to detect nucleases could be mainly divided into two categories. The first category that treats nucleases as any other gene or protein and detection is employing gene expression or protein expression methods. On the top of these techniques are the IHC, PCR and ELISA. The second category exploits the unique feature of nucleases which is the catalytic activity. Studies within the latter category are remarkably fewer, referring to the yet to be fully explored potential of harnessing nucleases as biomarkers that are detected by optimized specific and sensitive oligonucleotide substrates.

Paper II. Kinetic Screening of Nuclease Activity Using Nucleic Acid Probes

In this paper we propose a flexible approach for detection of disease associated nuclease activity using oligonucleotides as substrates. We describe design and selection of probes that target a certain nuclease activity with high sensitivity and specificity. In our approach, multiple rounds of screening for nuclease activity are performed. Based on the sequences of best performing probes at each round, a new generation of probes are designed with enhanced specificity and sensitivity. Sugar and chemistry (chemical modification type) preference by detected nuclease activity are considered. New probes are designed easily taking advantage of flexibility of nucleic oligonucleotides synthesis and chemical modification.
We have designed probes specific to a secreted nuclease activity by *Salmonella* applying the proposed approach. During the first screening a library comprised of natural DNA and RNA is used. Based on the results, only RNA-based probes were included as substrates. Fully modified sequences, or sequences with only modified purines or pyrimidines with 2'F or 2'OMe were used. Probes with 2'-OMe chemistry were suggested as most suitable substrates comparing nuclease activity profiles of *Salmonella* and control *E.coli* bacteria. As, such the protocol proposed in this paper suggests the potential of utilizing nuclease activity as a biomarker.

Paper III. Exploring Nuclease Activity Profile of Breast Cancer Cells

In the study provided in this paper, we aimed to explore nuclease activity associated with a panel of breast cancer cell lines with various molecular subtypes. In a previous work published by Hernandez et al, a specific nuclease activity associated with SKBR3 was observed towards 2'OMe and 2'F modified DNA probes. In the current study (paper III), a screening for nuclease activity of 9 breast cancer cells and 2 healthy counterparts was performed. As substrates, a library of 75 probes was used. Specific cell membrane associated nuclease activity for the breast cancer cell line BT474 was observed, which degrades the probe DNA PolyAT with a greater magnitude compared with healthy cell lines.

DNase but not RNase activity is a common feature between both breast cancer cell lines, however, towards different oligonucleotide probes.
Nuclease activity was assayed in presence of chelators EDTA, EGTA or NTA that sequester Ca^{2+}, Mg^{2+} and Fe^{3+}, respectively. Additionally, divalent metal cations such as Mg^{2+}, Ca^{2+}, Mn^{2+} and Cu^{2+} were also added individually to assess dependence of the nuclease on metal cations. Thermal analysis was also performed by incubating cell suspension at -80, -20, 4, 40, 60, 70 and 90ºC.

Paper IV. Discovery and Proof-of-Concept Study of Nuclease Activity as a Novel Biomarker for Breast Cancer Tumors

In this paper, we describe utility of nuclease activity as a biomarker to discriminae between healthy and cancer breast tumor tissues. FRET probes were designed and used as substrates in assaying nuclease activity of tissue homogenates.
Initially, screening of retrospectively collected 58 paired samples (29 cancer and 29 health tissue) was performed using a library of 12 probes. Three best performing probes were identified based on analysis results and integration of computational analysis. Additional 24 probes were designed based on the results of the first screening. Chemical modifications of 2'F or 2'-OMe were integrated in selected nucleotides along probes. The aim was to increase resistance of the second-generation probes against endogenous nucleases, and to increase the stringency to favor cancer associated nuclease activity. A cohort of 44 paired samples (22 cancer and 22 health tissue) were screened for nuclease activity against the 24 probes and a set of 3 probes was identified.
The promising 6 probes from the retrospective screenings were utilized in a prospective screening. Namely, 61 samples from patients were blindly screened and 3 probes : DNA, Poly A 2'-F DNA and AAACCCchi DNA were identified as the most efficient combination to distinguish cancer from healthy tissues. Together the 3 probes correctly predicted the status of 89% of the tumors, with 82% sensitivity and 94% specificity. Additionally, the probes show high stability in human serum. The data clearly highlight the potential of nuclease activity as a biomarker in breast cancer detection and suggest the set of 3 probes as an additional diagnostic toolkit to the histopathological analysis.

Paper V. Activatable MRI Probes for the Specific Detection of Bacteria

Although fluorescent activatable probes are very useful in research, their application *in vivo* and in clinical context is constrained by the background signal and limited tissue penetration. Activatable MRI probes, overcome these shortcomings by providing high spatial resolution, tissue penetration with no limit and high signal-to-noise (SNR) ratio. In this paper, we report on development of an activatable MRI-probe that is oligonucleotide-based, and specifically detects micrococcal nuclease secreted by *S. aureus*.
To generate this MRI-probe, TT-oligonucleotide that is specifically degraded by MN was coupled with D-SPION superparamagnetic quencher at the 5'-end. At the 3'-end, TT-probe was linked with a dendron functionalized with several gadolinium complexes, as enhancers. Initially, SPION confers quenching to Gd^{3+} *T1*-relaxation and the MRI-probe is "off" due to the proximity between SPION and Gd^{3+}. Only upon biorecognition and degradation of TT-oligonucleotide by MN secreted from *S. aureus*, MRI-probe is "switched-on" due to the increased distance between SPION and Gd^{+3}, and MRI-signal is amplified. This probe is useful for *S. aureus* detection with rapid, sensitive and specific manner that could be applied *in vivo* to detect not only bacterial infection, but also any disease with altered nuclease activity.

Chapter 8

8. Key Results

- Nucleases have been frequently reported as potential biomarkers for cancer diagnosis (paper I). FEN1, APE1, SND1, MRN complex, XPF/XPG, TREX2, DNase1, RNaseL and RNase1 in addition to global serum RNase activity are some but not all nucleases investigated in cancer (paper I).

- An alteration of nucleases expression (protein or mRNA), subcellular abundance, mutational status, and catalytic activity in cancer is observed (paper I).

- Research on harnessing nuclease activity in cancer detection deserves further efforts because there is a solid body of knowledge on the diagnostic potential of nucleases in cancer, and studies harnessing nuclease activity are relatively less present compared with those conventionally investigating gene or protein status (paper I).

- We were able to identify a nucleic acid probe specifically degraded by a bacterial nuclease activity (paper II). A library was rationally designed and utilized as substrate in assaying nuclease activity. Sugar substrate and chemical modification preference were observed, using first- and second-generation libraries, respectively.

- Implementation of nuclease activity as a biomarker for detection, screening and iterative probe design were proposed in the paper II.

- Screening for nuclease activity of breast cancer cell lines unraveled a specific nuclease activity associated with the BT-474 cells towards the oligonucleotide DNA PolyAT (paper III). BT-474 nucleases degrade DNA PolyAT with 2 folds greater magnitude than nucleases associated with BJ and CCD-18-Lu normal fibroblasts.

- The nuclease activity in paper III is membrane bound, sensitive to temperatures above 40ºC, but not compromised at as low temperature as -80ºC. The chemical analysis indicates a possible dependence on Mg^{2+}. And kinetic assay demonstrates an increased catalytic activity over the time of measurement, with already a 2 folds higher nuclease activity associated with BT-474 compared with the healthy BJ and CCD-18-Lu cells after 20 minutes incubation with the probe DNA PolyAT. Treating BT-474 cells with trypsin that disrupts cell surface proteins reduced the nuclease activity, demonstrating the cell surface bound nuclease activity.

- By screening of clinical samples of breast tissues (paper IV), we identified a set of 3 probes; DNA, Poly A 2'-F DNA and AAACCCchi DNA with a high potential to discriminate between cancer and healthy breast tumors. Together, these DNA-based probes were able to correctly identify the status of 89% of the investigated tumors with a sensitivity of 82% and specificity of 94%, compared with histopathological assessment. The set of 3 probes (paper IV) exhibited high stability in human serum, which indicates the validity of the probes for clinical use.

- The MRI-probe was characterized using TEM, DLS and UV-Vis (paper V). TEM visualized an ellipsoidal morphology of D-SPION with a size of about 20nm, while the size of activatable MRI-probe was up to 24nm. The increase in the size, which is about 4nm, is in line with coupling dendron-Gd^{3+} to the D-SPION. An average hydrodynamic size of 23.26nm and 34.85nm was determined by DLS for D-SPION and MRI-probe, respectively. Size measurement was consistent between TEM and DLS. Both methods confirmed the increase in thickness of MRI-probe compared with D-SPION due to coating with dendron and TT-probe. Additionally, hydrodynamic size distribution determined by DLS demonstrated no aggregation of MRI-probes. Successful integration of TT-probe with D-SPION and MRI-probe was demonstrated by UV-Vis that visualized peaks consistent with this incorporation.

- Relaxivity (r1) of dendron- Gd^{+3} was 3.66-fold greater than that of the MRI-probe (paper V), as determined after calculating the slope from 1/T1 for the measured entities, which confirmed a successful "magnetic quenching".

- The efficiency of the MRI-probe (paper V) in specific detection of bacteria was evaluated using an NMR MiniSpec that allows measuring T1-realaxation time. A series of concentrations of the probe incubated with media cultivated with target or control bacteria were tested, along with fresh media as buffer control. No significant change in T1-values was detected for the controls, while a clear shortening of T1 or elevated 1/T1 in a concentration dependent manner was reported for the *S. aureus* sample. The results confirm a specific recognition of bacteria by the MRI-probe.

- A specific detection of *S. aureus* by the MRI-probe was demonstrated with MR imaging (paper V). The images show an enhanced T1-based contrast in presence of MN compared with controls. Additionally, SNR calculation demonstrated a signal increase by 3.80 or 6.49 for *S. aureus* samples compared with the control *S. epidermidis* when 0.03 mM or 0.07 mM Fe concentration was used, respectively.

Chapter 9

9. Conclusion and Remarks

The need to diagnostic biomarkers for breast cancer is unsaturated, and hence diverse biomolecules are investigated for potential diagnostic value. Research has provided robust evidence on the potential utility of nucleases as diagnostic biomarkers in cancer. A differential status between healthy and cancer for many nucleases is demonstrated frequently, in multiple cancer types, and mainly by targeting the protein or gene expression of these enzymes. FEN1, APE1, RNase4 and RNase5 are few examples. This gave a rise to harnessing cancer associated nuclease activity as a biomarker towards tailored nucleic acid probes, that are detection tools in this context.

Our results have confirmed the applicability of nuclease activity in cancer detection in a specimen ranging from established breast cancer cell line to clinically valuable samples of breast tissues. Regarding cell lines, our results indicate that a nuclease activity associated with BT-474 breast cancer cells towards DNA PolyAT probe is distinctive feature for these cells that could be implemented to detect or identify this cell line in biological contexts. And more importantly, our results demonstrate the potential of breast cancer associated nuclease activity as a biomarker that discriminates between cancer and healthy tissue samples obtained from patients. The successful detection with high accuracy, sensitivity and specificity applied on valuable clinical samples suggests a facile translation into clinical use. A panel of 3 oligonucleotide probes for detecting breast cancer tumors was identified, and this strategy could be already implemented in the clinic as an adjunct detection toolkit to the mainstay of histopathological analysis. Intratumor heterogeneity is present in cancer with various cell subpopulations bearing characteristics that define diverse phenotypes. Heterogeneity includes genetic alteration, somatic copy number alteration and structural variation that is present at a different extent throughout a tumor. A global indicator, such as nuclease activity detected by the 3 probes, that distinguishes cancer tumor from healthy is therefore an added value to the diagnostic landscape. Additionally, detection of nuclease activity instead of targeting the protein or gene expression provides: 1) detection of nucleases with known or yet to be characterized expression in tumors or even proteins

with yet to be discovered nuclease domain. 2) Rapid detection without required amplification step. 3) Detection tools with high reproducibility and animal-free production.

Furthermore, our results indicate that disease associated nucleases could be detected with specific nucleic acid probes beyond FRET based system, namely by MRI. This is of a special interest as it sets up the stage to develop breast cancer probes from the proposed initial use (as an additional diagnostic toolkit) into a noninvasive diagnosis activatable MRI-breast cancer probes. We envision that activatable MRI-probes could be sensitive and specific cancer detection tools. They combine the benefits of unlimited tissue penetration, high spatial resolution and non-invasive approach of MRI, with the high signal-to-background ratio achieved by the "switchable" activatable probes. Therefore development of MRI-activatable probes holds a great advantage for early cancer detection and clinical management and deserve considerable efforts [228].

Although our current results do not identify nucleases behind a detected catalytic activity, it goes hand in hand with many reports that indicate alteration in nucleases in cancer. Importantly, our approach proposes a robust platform of screening for nuclease activity associated with any pathological conditions. A rational probe design and refining based on multiple screening rounds enable tailoring nucleic acid probes for detecting diseases. Such a platform allows identifying substrate preference and favorable chemical modification to identify specific nucleic acid probes that target known or yet to decipher nucleases in a disease of interest.

Chapter 10

10. Future Aspects

At the current moment, probe design relies on results analysis and decision making by researchers with try and error manner being an essential part of design improvement. A future aspect is to implement artificial intelligence in result analysis and design guidance or next generation probe sequence suggestion. Time and material saving are among the benefits in this future quest.

Studies are performed on cell lines and tumors. Nipple aspiration fluid, ductal lavage, random peri-areolar fine needle aspiration or peripheral blood/serum are other relevant clinical samples to investigate for future diagnostic applications.

Further design refining of the probes increases the sensitivity and specificity, enabling using the cancer-probes as drug delivery system to treat breast cancer. Such a drug delivery platform provides an immense benefit of decreasing side effects of a variety of therapeutic agents.

The nucleic acid probe sequences, as previously mentioned, are biorecognition molecules. Simply put, the compatibility with an imaging modality is dependent on the contrast agents they are coupled with. Utilizing the compounds provided by state of art nanotechnologies, it is possible to implement nucleic acid probes in multimodal contrast agents that could be used in several imaging techniques [229].

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