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HYPMED

Digital Hybrid Breast PET/MRI for Enhanced Diagnosis of Breast Cancer

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D 4.3 Definition of biomarker signatures correlated to PET–MR imaging

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1 Introduction

Project summary

Since the introduction of systematic mammographic screening, the incidence of *ductal carcinoma in situ* (DCIS) has increased by more than 5-fold. Currently, guidelines recommend at least local treatment for all DCIS similar to that of invasive cancers. However, it is well established that a substantial proportion of DCIS will not progress to invasive cancer even if left untreated. Unfortunately, we are currently unable to predict which DCIS will progress, causing considerable overtreatment. Accordingly, there is a substantial clinical need for prognostic information on DCIS that helps predict whether a given DCIS will be able to progress to invasive cancer. Class II tumor suppressor genes (C2TSGs) inactivated by promoter DNA hypermethylation are important and early players in breast cancer initiation and progression and may constitute helpful biomarkers for assessing the risk of progression. Within the HYPMED project, we have thus developed a novel strategy how prognostic C2TSGs may be applicable for DCIS risk classification.

Background

Most malignant cases of human breast cancer belong to the group of invasive *ductal carcinoma* (IDC) – nowadays referred to as “no special type” (NST). IDC develops from the epithelial lining of the milk ducts, a pre-invasive stage called *ductal carcinoma in situ* (DCIS). For unknown starts to progress by penetrating the basal lamina, thus invading into the surrounding breast tissue. However, it is not clear which DCIS will progress and which DCIS will stay silent (indolent). Pathologists have defined risk classes depending on purely morphological cell appearance and necrosis pattern (see Figure 1), classifying DCIS into three classes, low-grade DCIS, intermediate grade DCIS and high-grade DCIS. This classification system is helpful but not sufficient to determine a personalized treatment protocol for the individual breast cancer patient.

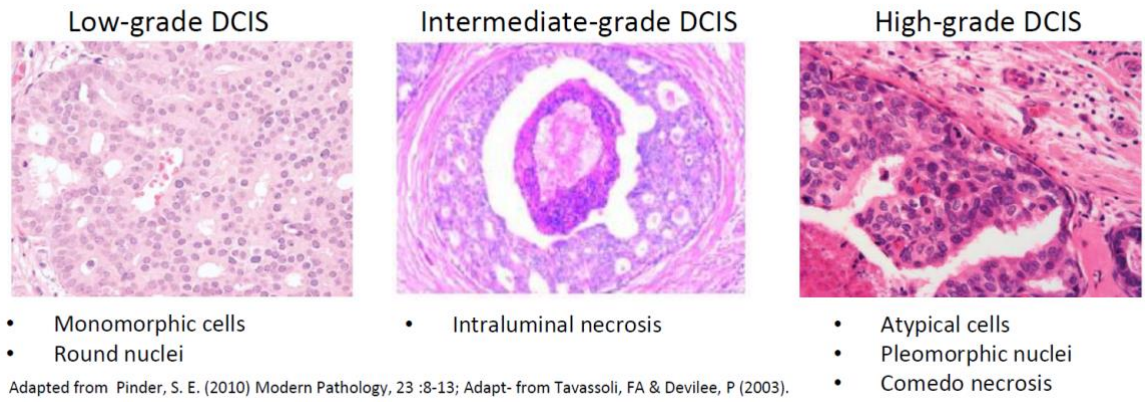


Figure 1: Current pathological classification of *ductal carcinoma in situ* (DCIS)

Thus, more advanced molecular biomarkers have been developed for a more precise risk classification of DCIS. Epigenetic biomarkers like DNA methylation markers are suitable molecular markers since promoter DNA methylation in breast cancer is both an early and frequent event, thus potentially ideal conditions for a comprehensive biomarker signature composed of a handful of promoter DNA methylation markers.

Beyond pathological methods, there is reason to assume that the imaging phenotypes of DCIS can also provide prognostically useful information, and possibly help classify the future behavior of DCIS. Not all DCIS do exhibit enhancement on MR imaging, or enhancement on PET. Enhancement does not clearly correlate with existing classification systems of DCIS.

DCIS enhancement on MR imaging might provide prognostically useful information because for one, it demonstrates that a DCIS does interact with the world outside the milk ducts, and did manage to successfully promote peri-ductal angiogenesis, which is the essential basic prerequisite of enhancement on MR imaging. For another, since we observe intra-ductal, and not (only) peri-ductal enhancement in DCIS on MR imaging, there appears to be a transition of contrast enhancement from the peri-ductal into the intraductal space. This is noteworthy since the normal milk duct wall has tight junctions that effectively prevent any exchange of larger molecules across the wall, i.e. from the peri- into the intraductal space and back; thus, there is never contrast agent accumulation in a normal milk duct since gadolinium chelate contrast agents are very large, complex molecules. However, in DCIS, there is obvious transgression of the contrast agent into the intraductal space. This suggests that, in DCIS that do show enhancement on MR imaging, there is a pathologically increased permeability of the milk duct wall at the site of the DCIS, which, in turn, suggests the release of protease by the intra-ductal cancer that leads to a local degradation of the ductal barrier.

Although these pathophysiological considerations are attractive thought for further classification of DCIS, it is difficult to prospectively investigate the accuracy with which we can classify the future behavior of DCIS by the respective MR imaging phenotype because this would require a prospective clinical study where, in women with DCIS without enhancement, would not undergo treatment, but watch-and-wait. So far, this has not been put to practice for several ethical issues.

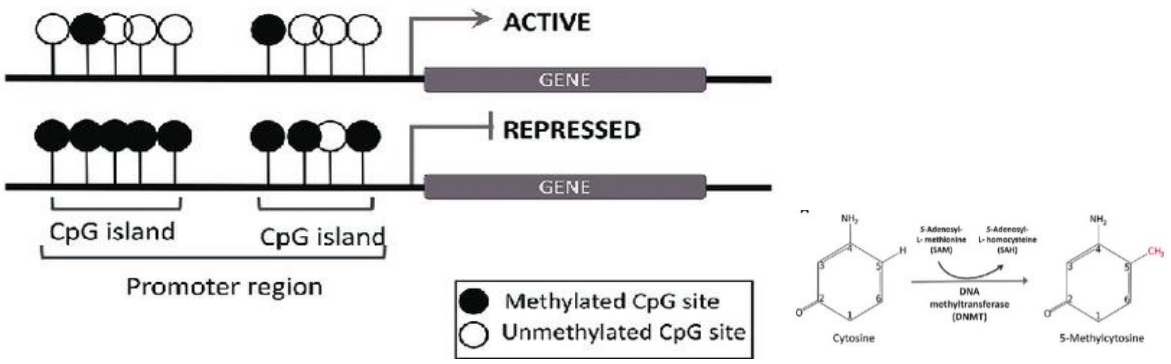
Accordingly, and in the absence of accurate prognostic information based on current pathological markers, we need to further investigate the prognostic power of MR and PET imaging of DCIS by correlating the respective imaging phenotype with prognostic information that is available by novel genomics- or epigenetics based pathological methods. This is the aim of this work package.

2 Definition of biomarker signatures

2.1 Introduction

Background:

The breast cancer epigenome exhibits characteristic changes in global DNA methylation, such as the promoter DNA hypermethylation of tumor suppressor genes and hypomethylation in other genomic regions like repetitive elements. Unlike Class I tumor suppressor genes, that are being inactivated by permanent DNA alterations (mutation, chromosomal deletion), class II tumor suppressor genes are inactivated by epigenetic processes, that lead to loss of expression and are reversible, in principle. Class II tumor suppressor genes have been already defined by Ruth Sager and coworkers in the 1990 years, which we will abbreviate as "C2TSGs" in the following. Since tumor specific promoter DNA methylation of C2TSGs is considered the last and best tangible step leading to permanent transcriptional shut down and thus to the loss of the tumor suppressive function (see Figure 2).



Adapted from Alarcon A. et al. Gastric carcinoma, 2017 and Illingworth R.S. et al, FEBS Letters, 2009.

Figure 2: Silencing of Class II tumor suppressor genes (C2TSGs) by promoter DNA hypermethylation at so-called CpG sites (lollipops) which are usually arranged in so-called CpG islands. DNA methylation at the C5-position of cytosines within CpG duplets is catalyzed by DNA methyltransferases (DNMTs). The silenced (repressed) gene can no longer be transcribed. The process is in principle reversible.

Tissue collections:

In Aachen, a tissue collection of pre-invasive breast lesions (*ductal carcinoma in situ* or DCIS) with sufficient pathological and clinical data (n=118) has been characterized, being composed of DCIS high

(n=59), DCIS intermediate (n=43) and DCIS low (n=16) and in addition normal breast tissues (n=30) as reference material.

Methods:

Identification and filtering procedures for novel putative C2TSGs from the Cancer Genome Atlas (TCGA) database were described in our last years "Deutsche Gesellschaft für Pathologie" (DGP) annual meeting contribution by Dehelean et al. (see: Dehelean DC et al. (2021): Identifying novel class II tumor suppressor genes in breast cancer. In: Virtuelle Pathologietage der Deutschen Gesellschaft für Pathologie. Abstract 2021: p106). In short, Gene expression data (RNA-Seq) for 1102 breast cancer samples along with 113 normal breast tissue samples was downloaded from the Cancer Genome Atlas (TCGA) database. Raw counts were normalized using the trimmed mean of M-values (TMM) method and differential gene expression (DGE) analysis was performed using Limma-Voom. To adjust for multiple testing the Benjamini and Hochberg method was used. 450k methylation array, expression, and survival data were acquired from Xenabrowser based on TCGA breast cancer data.

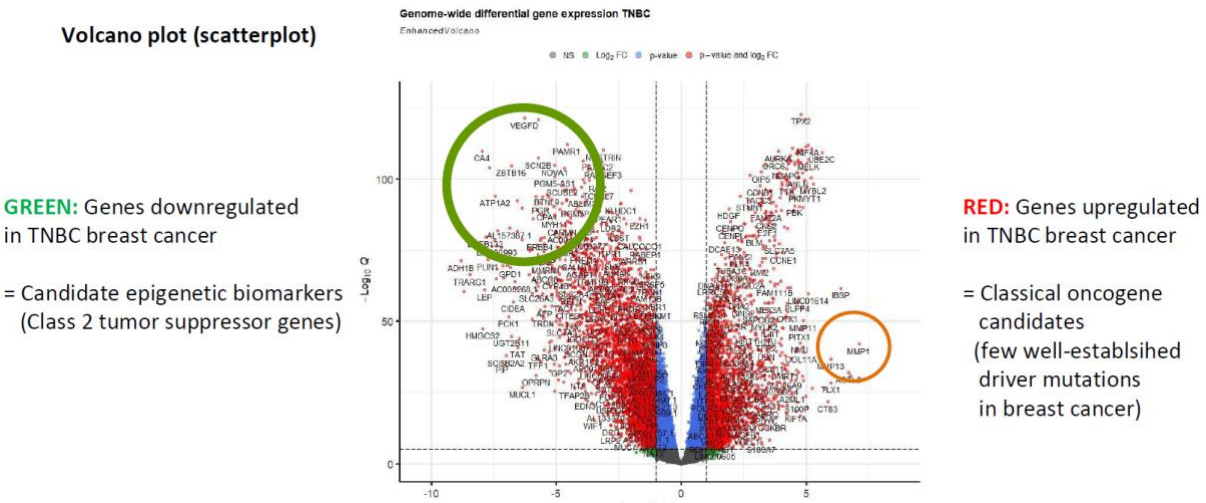


Figure 3: Discovery of novel class II tumor suppressor genes (C2TSGs). Volcano plot analysis is shown for genes downregulated (left side GREEN) in triple negative breast cancer (TNBC) which are candidate epigenetic biomarkers.

Candidate class II tumor suppressor genes were selected for further analysis based on the following criteria: a) association of gene expression with longer overall survival, b) genes containing definite CpG islands in their proximal promoter region and c) genes encoding proteins with known involvement in important signaling pathways of the cancer cell. This finally led to the definition of 12 novel putative class II tumor suppressor genes (see Figure 4).

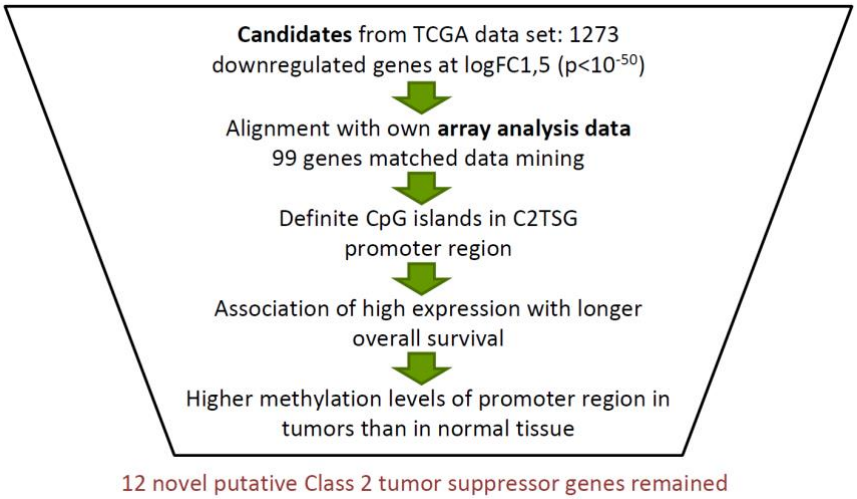


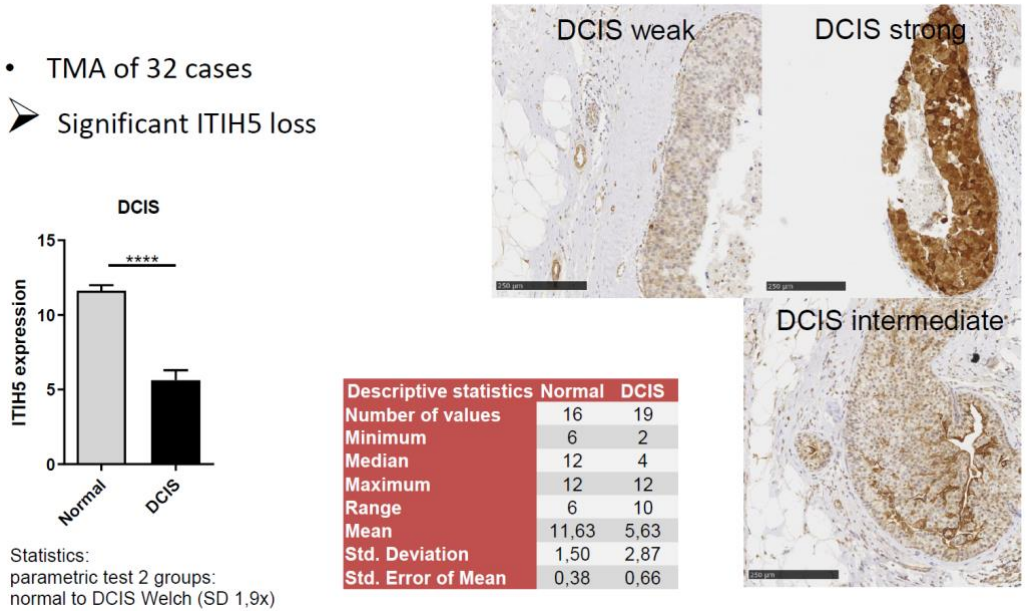
Figure 4: Filtering process used to define the most promising candidate class II tumor suppressor genes (C2TSGs). Of initially 1273 candidate genes 12 were chosen for further analysis.

Molecular classification of these C2TSGs according to the intrinsic breast cancer subtypes was performed using the webinterface RosettaSX (www.rosettasx.com). Loss of expression of the proteins encoded by C2TSGs was analyzed by immunohistochemistry using tissue microarrays generated from 143 DCIS identified from Aachen and Münster. DCIS expression patterns were compared to pathological and clinical data using SPSS.

Results:

<u>New putative DCIS breast cancer biomarker ITIH5</u>

Our research group was the first to describe that ITIH5 is a class II tumor suppressor gene (C2TSG) in human breast cancer. ITIH5 promoter methylation was highly associated with a shortened recurrence-free (p=0.0045) and overall survival (p=0.008) of breast cancer patients. Interestingly, ITIH5 promoter methylation was also strongly associated with both lymph node and long-distance metastases (p=0.003 and p=0.047 respectively), which clearly supports our working hypothesis that ITIH5 is a novel metastasis suppressor gene of breast cancer. However, if loss of ITIH5 expression in early breast cancer like DCIS contributes to DCIS progression thus being a potential biomarker candidate is still unclear and was further investigated in the HYPMED project. We found in a larger collection of DCIS that ITIH5 expression is significantly downregulated in DCIS compared to normal breast epithelium (see Figure 5). Analysis of DCIS ITIH5 expression in comparison to patient clinical data is still underway.



Descriptive statistics	Normal	DCIS
Number of values	16	19
Minimum	6	2
Median	12	4
Maximum	12	12
Range	6	10
Mean	11,63	5,63
Std. Deviation	1,50	2,87
Std. Error of Mean	0,38	0,66

Figure 5: Loss of ITIH5 expression in DCIS compared to normal breast tissue. ITIH5 protein expression in DCIS demonstrate a considerable variability, potentially associated with risk of DCIS progression.

New putative DCIS breast cancer biomarker SCN4B

An interesting putative novel breast cancer biomarker we found within the HYPMED project is SCN4B (Hilgers L et al. (2021): Epigenetic regulation of the putative breast cancer metastasis suppressor gene. Annals of Oncology 32 Sup2 S69). SCN4B is a protein that is expressed in DCIS but also relevant later in breast cancer development for suppressing metastasis. Analysis of DCIS SCN4B expression in comparison to patient clinical data is still underway.

New putative DCIS breast cancer biomarker IRX1

IRX1, also known as Iroquois homeobox protein 1, has not been associated with breast cancer previously but was also newly identified by our discovery approach within the HYPMED project. The IRX1 gene has a CpG rich promoter region typically for a class II tumor suppressor genes (C2TSG). DNA hypermethylation of this promoter is the likely cause of IRX1 loss of protein expression, seen in a high percentage of breast cancer cases (an example shown in Figure 6). Analysis of DCIS IRX1 expression in comparison to patient clinical data is still underway.

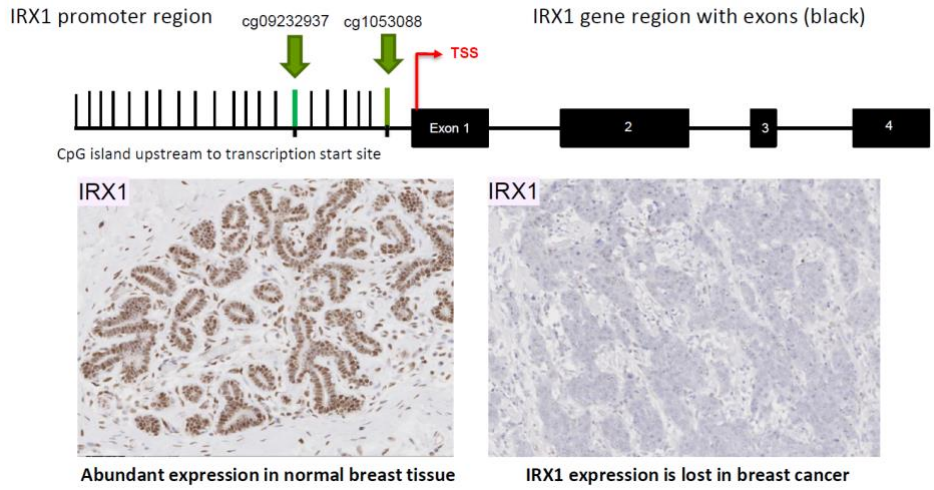


Figure 6: Loss of IRX1 expression in breast cancer (*invasive ductal carcinoma*) compared to abundant expression in normal breast tissue. This is the first described association between IRX1 and breast cancer.

Finding that class II tumor suppressor genes can be grouped according to intrinsic molecular subtypes

A very interesting result from the HYPMED project WP4 part was the finding that epigenetically silenced tumor suppressor genes (C2TSGs) in invasive breast cancer samples can be broken down in three subgroups showing loss of expression in accordance with the well-known intrinsic molecular subtypes. These C2TSG groups we termed "**M**olecular **S**ubtype **S**pecific **L**oss" or MSSL. While genes belonging to MSSL1 (group 1) are lost in TNBC and luminal B tumors, genes belonging to MSSL2 (group 2) are only lost in TNBC, and those belonging to MSSL3 (group 3) are only lost in luminal B tumors. Analyzing protein distribution of members of each group in DCIS revealed strikingly different expression patterns, indicating that such epigenetic features may provide prognostic information about DCIS and their potential regarding progression to invasive cancer.

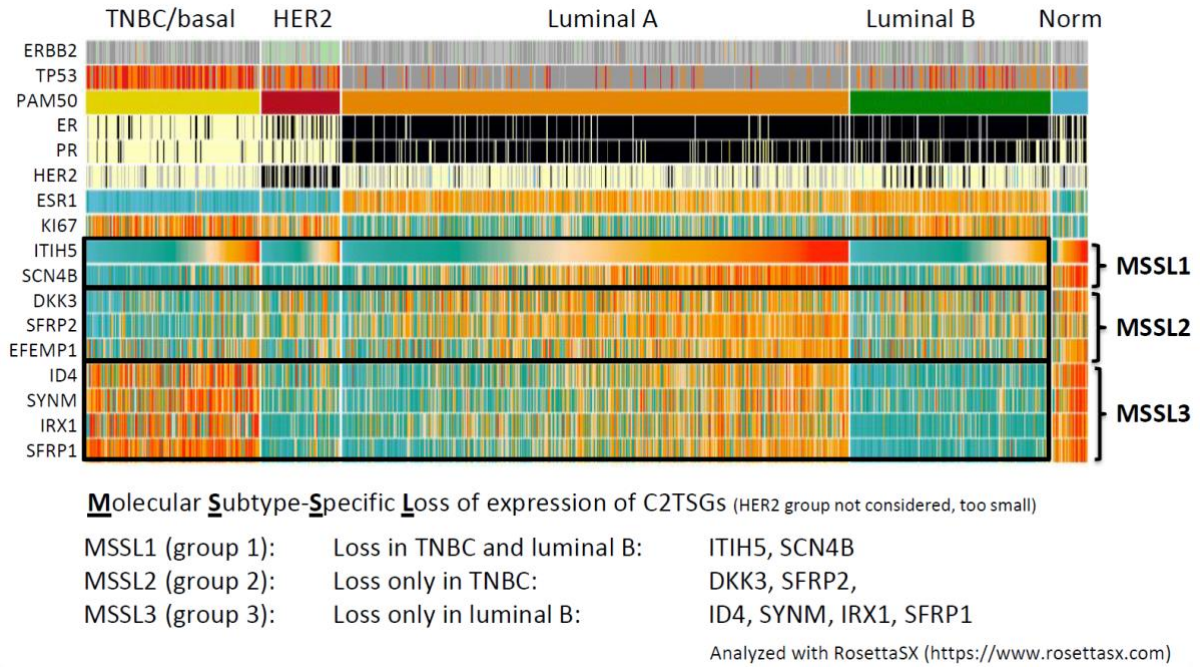


Figure 7: Class II tumor suppressor genes (C2TSGs) can be grouped according to the well-known intrinsic molecular subtypes of breast cancer, here with regard to loss in luminal B (group 3), loss in TNBC (group 2) or loss in both luminal B and TNBC (group 1).

A molecular classification of DCIS according to expression loss of epigenetically silenced tumor suppressor genes (C2TSGs) in line with potential later intrinsic molecular subtypes may be helpful for further DCIS risk classification and thus future therapy stratification. Thus, we are now defining new epigenetic biomarkers that can stratify low aggressive (non-invasive) DCIS from those aggressive DCIS tumors with a high potential to get invasive.

3 Correlation to PET–MR imaging

3.1 Introduction

Due to the pandemics and other challenges encountered during the engineering phase of the new HYPMED device, the project suffered from multiple delays – with the result that the finalization of the device was delayed, and thus, the new MDR came into effect before the clinical trial could be started. As a result, with the new regulatory framework, the actual clinical trial could not be performed, and thus no tissue samples of patients undergoing imaging with the HYPMED device could be collected for correlation with immunoscores and biomarkers. For this reason, we changed the approach to still achieve results with relevance for the project. We were able to establish a cooperation with the Department of Pathology in Vienna University. After all requirement were met that regulate the shipping of biosamples across EU countries, the Department of Pathology of the University of Vienna

was able to provide us with pathology tissue specimen from the women who had undergone conventional, whole-body contrast enhanced FDG-PET/MR imaging within WP3 (see Deliverable D4.2 for further information).

We are planning to validate our novel DCIS methylation biomarkers in future research projects with the team of Münster pathology and Aachen radiology.

4 Project summary and outlook

Project summary and Outlook

There is an urgent clinical need for accurate prognostic information on a given DCIS, to avoid overtreatment (i.e. treatment of DCIS which, even if left undiagnosed and/or left untreated, would not progress to invasive disease), as well as undertreatment (i.e. failure to appropriately treat a DCIS which, if left undiagnosed and/or left insufficiently treated, would progress to an invasive cancer that would cause morbidity and mortality of women). There is good reason to assume that imaging phenotypes of DCIS on MR imaging, and even more so on PET/MR imaging, will provide prognostically useful information that could be exploited for treatment stratification of women with DCIS – and that do provide this information non-invasively, without biopsy, and thus also provide information that is indeed representative for the entire DCIS. To further investigate this, we need improved pathophysiological methods that help validate MR and PET/MR imaging findings of DCIS. Class II tumor suppressor genes (C2TSGs) inactivated by promoter DNA hypermethylation may be the ideal candidate for this purpose, because they are important and early players in breast cancer initiation and progression and may constitute helpful biomarkers for assessing the risk of progression. Within the HYPMED project, we have thus developed a novel strategy how prognostic C2TSGs may be applicable for DCIS risk classification.

A molecular classification of DCIS according to expression loss of epigenetically silenced tumor suppressor genes (C2TSGs) in line with potential later intrinsic molecular subtypes may be helpful for further DCIS risk classification and thus future therapy stratification. Thus, we are now defining new epigenetic biomarkers that can stratify low aggressive (non-invasive) DCIS from those aggressive DCIS tumors with a high potential to get invasive.