Topic: PHC-11-2015:
Development of new diagnostic tools and technologies: in vivo medical imaging technologies

HYPMED

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

Grant Agreement Number: 667211

D 4.1 Definition of different types of immune infiltrates: immunoscore

Lead Partner:	UK Münster		
Author(s):	WP4 partners		
Work Package No:	4		
Estimated delivery date:	M24 (December 31, 2017)	**Actual delivery date:**	M47 (November 20, 2019)
Nature:	Report		
Dissemination level:	Public		

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1 Summary

Different components of breast lesions account for different behaviour in imaging procedures. Especially microenvironment including inflammatory cells such as lymphocytes and macrophages but also fibroblasts and vessels in preinvasive and invasive tumours might contribute to distinctive features in MRI.

Inflammatory cells are known to play a role in microenvironment and are associated with prognostic implications. To characterise patterns of the inflammatory infiltrate that may lead to a specific imaging signature, UKM investigates this infiltrate by means of histology, immunhistochemistry and immunfluorescence in different benign and malignant breast lesions.

As a first step, a retrospective control group is being generated to establish a panel of antibodies to characterize the inflammmatory cells. Besides the analysis of the inflammasome in serial sections, the OPAL-system for multispectral immunfluorescence allows to analyse the interconnection of the different cellular components in the same slide. For this, the staining procedures are being established.

In a second step, the results from the retrospective series (with more than 150 cases) are used to develop an immunoscore and to correlate the results of histologic samples from the different lesions with patterns identified by PET/MRI procedures and with clinical/ prognostic relevance.

2 Introduction

Inflammatory cells are a component of tumor microenvironment, which is probably contributing as a key factor to the progression from in-situ to invasive carcinoma and may also promote the progression of localized to metastatic invasive breast carcinomas. Tumor-infiltrating immune cells such as different types of lymphocytes and macrophages may interrupt the immune balance of the body during the development, progression and dissemination of breast cancer. As possible mechanisms cancer cells influence and modify the cellular components of the intra- or peritumoral micromilieu resulting in immunosuppression or immunoresistance mechanisms (Alkatout et al. 2017). The positive impact of CD3- and CD8-positive lymphocytes on prognosis in breast cancer has been demonstrated in large cohorts of estrogen receptor-negative and triple-negative tumors (Shah et al. 2012). The positive effect of CD3-positive cells in prognosis of breast cancer has also been noted in a large systematic review of Mao (Mao et al. 2016). The central role of CD8-positive lymphocytes on recurrence-free and cancer-specific survival was confirmed in multivariate analysis by Miyashita (Miyashita et al. 2015). As evidence of the prognostic relevance of tumor-infiltrating lymphocytes

(TILs) was growing in the past years, Salgado et al. published recommendations from an International TILs Working Group in 2015 to give practical support for future studies. The group recommended to use HE stained slides to count TILs although it is well recognized by the group that it might be even more useful to use immunohistochemistry to characterize the complex network of immune cells further. The following figure 1 shows potential important cellular players in tumor microenvironment.

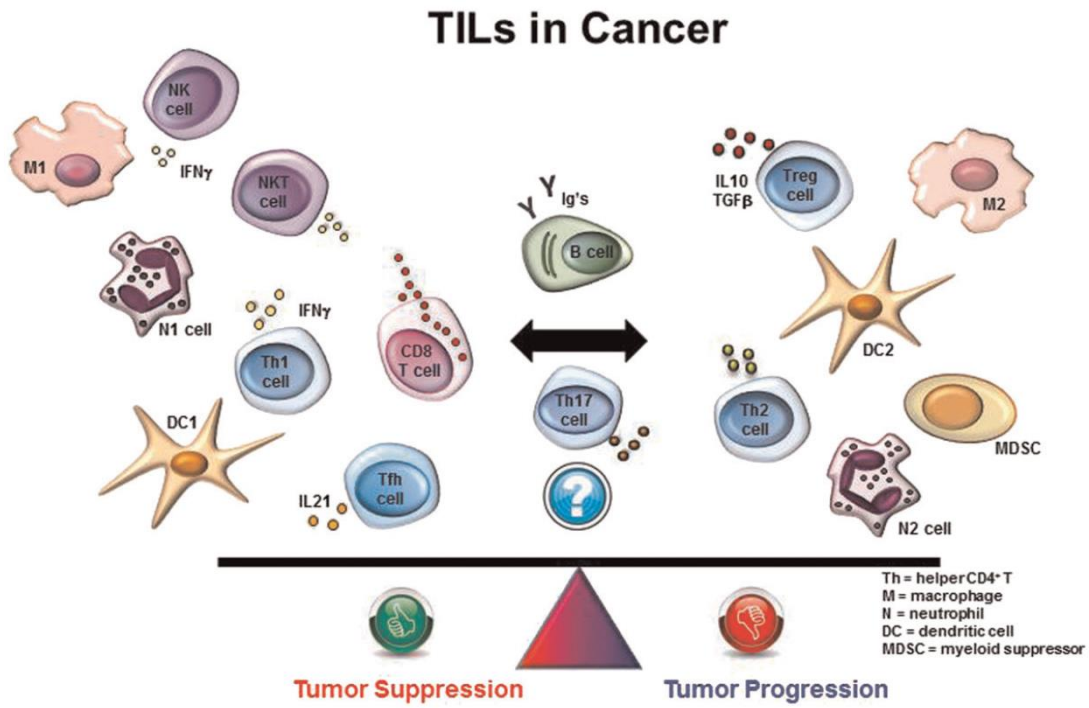

Figure 1. Potentially relevant cellular components in the microenvironment of tumors (acc. to Slagado et al. 2015)

As microenvironment might also contribute to different enhancement profiles of breast lesions in MRI, one aim of our work package is to characterize the immune infiltrate of different lesions to correlate specific signatures to PET/MRI imaging subtypes and to optimize the radiological settings of this new modality in a clinical context. Whether the different composition of immune infiltrates allows also an earlier detection of in-situ or invasive carcinomas will have to be demonstrated.
According to the working plan of our collaborative group (see in Figure 2) the set-up of the biorepository starts after year 2 beginning at the time point of this report.

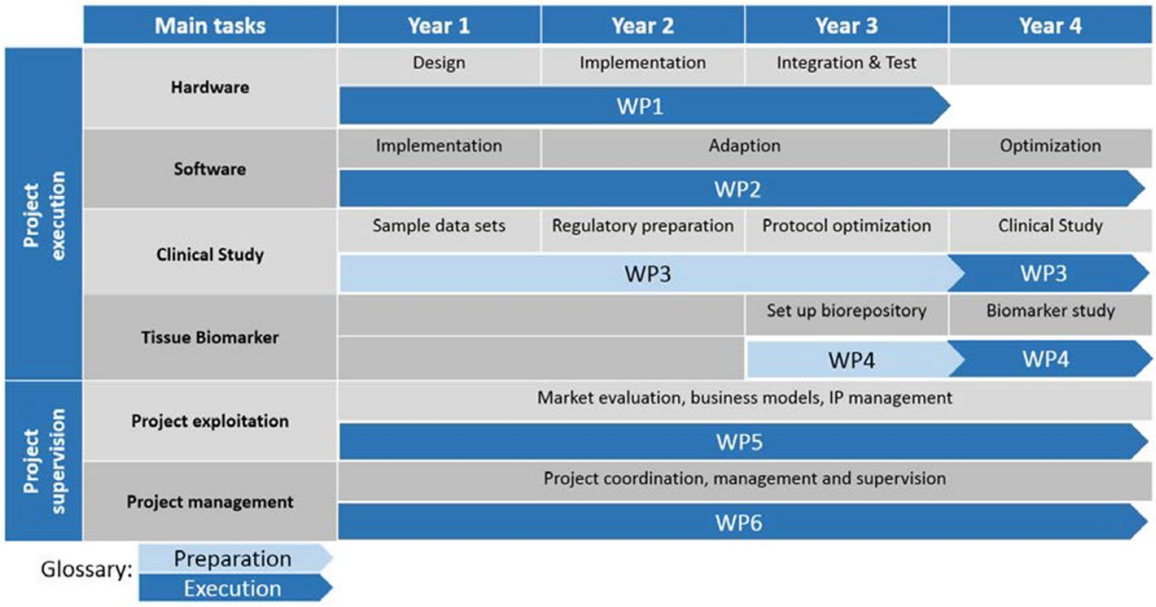

Figure 2. Working plan of HYPMED

3 Material and Methods

UKM used the first two years to generate a control series of different breast lesions, including 49 invasive carcinomas , 30 DCIS and a group of benign lesions containing 9 papillomas, 25 cases with ductal hyperplasia, 7 fibroadenomas, 30 radial scars and 3 cases of scleradenosis and lobular neoplasia. Antibodies against CD3, CD4, CD8, CD68, CD163, FoxP3, PD-L1 and PD-1 for conventional immunhistochemical stainings are planed to be stained. As first tests to establish a FoxP3 staining were not satisfying, we decided to use another clone which is being tested.

The Opal-System has beed purchased (see Figure 3) and a panel including CD3, CD8, FoxP3, CD163, and PD-L1 is currently being established.

Figure 3: Multispectral fluorescence microscope (OPAL; Perkin Elmer)

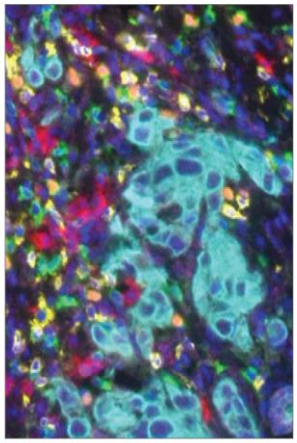

Figure 1. Breast cancer tissue section, Opal 7-color IHC. Red: CD20. Yellow: CD8. Green: CD4. Magenta: PDL1. Cyan: cytokeratin. Orange: FoxP3. Blue: nuclei.

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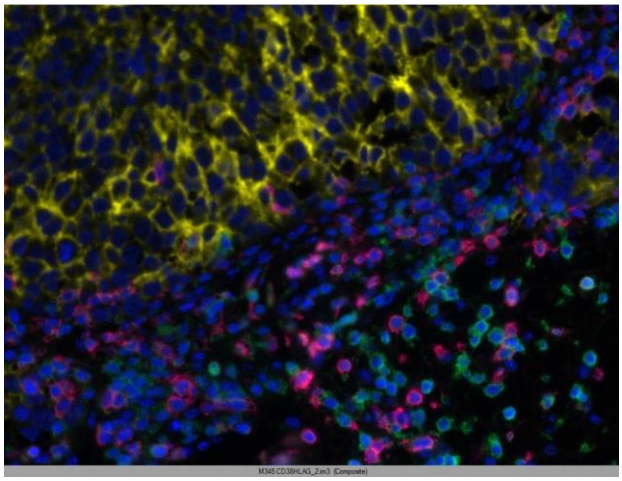

Ewing sarcoma: yellow = HLAG, green = CD3, red = CD8, blue = nuclei

Figure 4. Examples for successful simulateous visualisation of different cell populations on the same slide by the OPAL system (breast cancer left side, Ewing sarcoma right side)

Multispectral fluorescent immunohistochemistry allows to analyze connections between different components of the tumor microenvironment as shown in Figure 4.
To evaluate the retrospective and prospective samples in a standardised approach a first set of 10 cases of invasive carcinomas (NST) will be evaluated on full slides to compare the results with data generated on tissue microarrays (TMA). Measurements of the different components will be done in different areas of the lesion, i. e. in the center of the tumor and at the invasive margin. Special attention will be payed to the question whether these cells are located between the epithelial cells which may indicate the expression of neo-antigens on the cell surface or only in the stroma. By using digital image analysis we will be able to quantify the different non-tumorous cellular components in a standardised way and express the results in cells/mm² (Figure 5). This approach will allow us to compare our data with published data sets in breast cancer. As tumor heterogeneity may influence the results selection of the areas of interest for evaluation will be performed by two independent experienced pathologists (DH, EW).

Immune contexture		
Type	CD3, CD8, CD45RO	**Immunoscore**
Density	Cells/mm^2	
Location	Centre, Margin	
Orientation	Th1, cytotoxic, chemokines, adhesion	

Figure 5. Immunoscore to evaluate different types of breast lesions in a standardised approach using digital image analysis (acc. to Galon et al, 2014).

Furthermore, we will apply the recommendations of the International TILs Working Group to make data comparable (Figure 6).

Table 2. Recommendations for assessing tumor-infiltrating lymphocytes (TILs) in breast cancer

1) TILs should be reported for the stromal compartment (=% stromal TILs). The denominator used to determine the % stromal TILs is the area of stromal tissue (i.e. area occupied by mononuclear inflammatory cells over total intratumoral stromal area), not the number of stromal cells (i.e. fraction of total stromal nuclei that represent mononuclear inflammatory cell nuclei).
2) TILs should be evaluated within the borders of the invasive tumor.
3) Exclude TILs outside of the tumor border and around DCIS and normal lobules.
4) Exclude TILs in tumor zones with crush artifacts, necrosis, regressive hyalinization as well as in the previous core biopsy site.
5) All mononuclear cells (including lymphocytes and plasma cells) should be scored, but polymorphonuclear leukocytes are excluded.
6) One section (4–5 μm, magnification ×200–400) per patient is currently considered to be sufficient.
7) Full sections are preferred over biopsies whenever possible. Cores can be used in the pretherapeutic neoadjuvant setting; currently no validated methodology has been developed to score TILs after neoadjuvant treatment.
8) A full assessment of average TILs in the tumor area by the pathologist should be used. Do not focus on hotspots.
9) The working group's consensus is that TILs may provide more biological relevant information when scored as a continuous variable, since this will allow more accurate statistical analyses, which can later be categorized around different thresholds. However, in daily practice, most pathologists will rarely report for example 13.5% and will round up to the nearest 5%–10%, in this example thus 15%. Pathologist should report their scores in as much detail as the pathologist feels comfortable with.
10) TILs should be assessed as a continuous parameter. The percentage of stromal TILs is a semiquantitative parameter for this assessment, for example, 80% stromal TILs means that 80% of the stromal area shows a dense mononuclear infiltrate. For assessment of percentage values, the dissociated growth pattern of lymphocytes needs to be taken into account. Lymphocytes typically do not form solid cellular aggregates; therefore, the designation '100% stromal TILs' would still allow some empty tissue space between the individual lymphocytes.
11) No formal recommendation for a clinically relevant TIL threshold(s) can be given at this stage. The consensus was that a valid methodology is currently more important than issues of thresholds for clinical use, which will be determined once a solid methodology is in place. lymphocyte-predominant breast cancer can be used as a descriptive term for tumors that contain 'more lymphocytes than tumor cells'. However, the thresholds vary between 50% and 60% stromal lymphocytes.

Figure 6. Recommendations for assessing tumor-infiltrating lymphocytes (TILs) in breast cancer (acc. to Salgado et al. 2015)

4 Results

From the beginning of the project to the end of year 2 we selected more than 150 cases of benign and malignant breast lesions which typically occur in cohorts of women sent to MRI for breast evaluation. These lesions have been used to establish a panel of antibodies both in conventional immunohistochemistry and in our novel labelling system (OPAL) using fluorescent antibodies in the same slide to analyse interactions between immune cells. Stainings are ongoing an will be completed in the next months to be evaluated systematically.

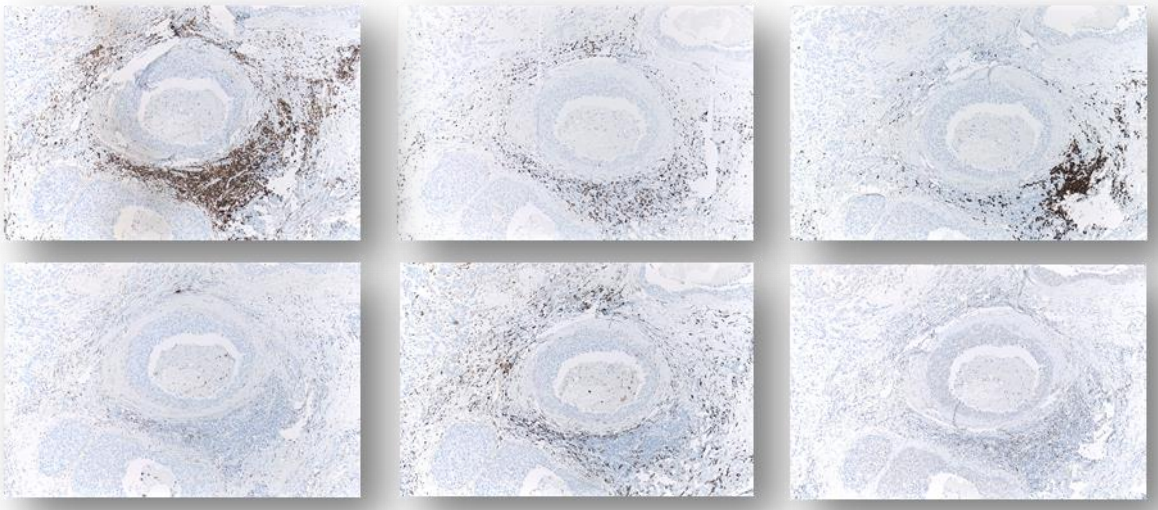

Figure 6. Different amounts of inflammatory cells in an intraductal carcinoma (high grade): CD4, CD8, CD20, CD68, CD163, PD-L1 (from left to right; original magnification 50x). Note the periductal accumulation of a mixed lymphocytic infiltrate composed of B- and T-lymphocytes.

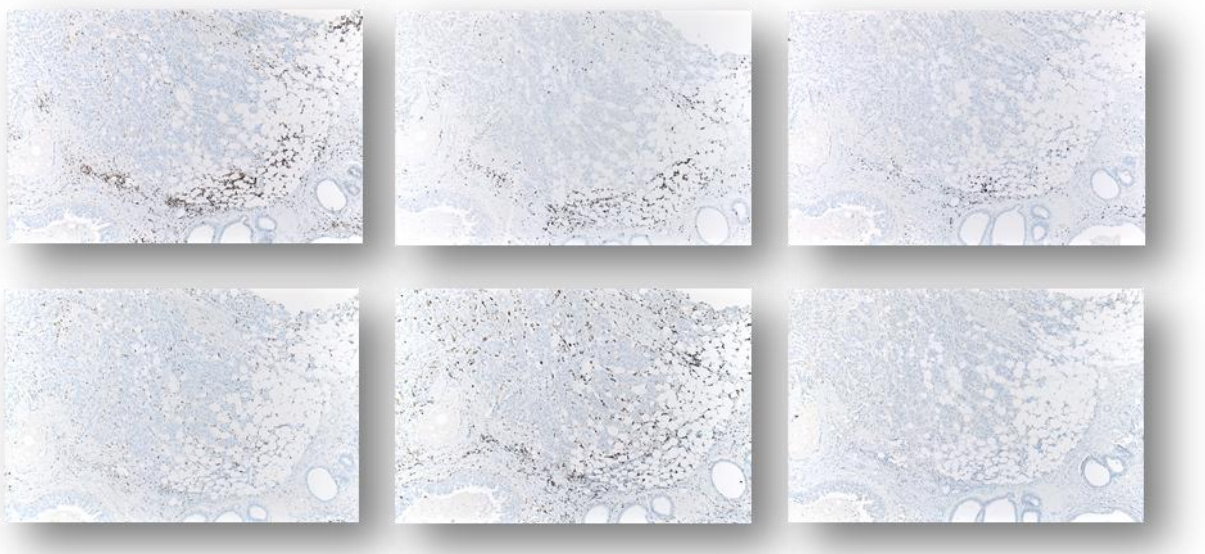

Figure 7. Different amounts of inflammatory cells in the invasive carcinoma component): CD4, CD8, CD20, CD68, CD163, PD-L1 (original magnification 50x). Note the accumulation of a T-cell dominated lymphocytic infiltrate.

In a second step vasculature of the tumors will be evaluated by quantifying vessel density in different lesions using antibodies against endothelial cells such as CD34, CD31 and ERG. Also these stainings will soon be completed.

5 Discussion/Relevance for the other WPs

In the past, several studies demonstrated the relevance of the tumor microenvironment and its cellular components for prognosis and treatment prediction in specific subtypes of breast cancer. High TIL levels in the stroma of triple negative breast cancer correlate with a better outcome after adjuvant anthracycline-based chemotherapy. In Her2+ disease higher TILs in baseline samples resulted in higher responses to trastuzumab in the FinHER trial (Loi et al. 2014). Taken together, there is increasing evidence for the importance of determinating the inflammasome in breast cancer. From genomic and epigenetic studies evidence grows that the biological behavior of different kinds of breast cancer is highly diverse. There might be even carcinoma subtypes without any biological relevance for the patient. One major goal of radiologic imaging of the breast is to predict the biological behavior of a specific lesion to prevent women from overtreatment.

It is very likely that distribution and density of immune cells as well as vasculature are influencing the presentation of different breast lesions in imaging procedures such as MRI and PET/MRI. By correlating the different types of immunoscores with the images we hope to get new insights into the predictability of a specific lesion in the breast to decrease operations in the future.

Our results will be also relevant for the second part in our work package 4 as in this part epigenetic profiles will be generated. Of course, the results are also highly influenced by the amount of inflammatory cells in a given tumor sample so we will have the unique possibility to correlate immunoscore, epigenetic profile and imaging in our collaborative working group. We expect that the conclusions will be of high relevance for all other work packages.

6 Conclusions and Achievements

During the first two years of our project we were able to select more than 150 cases of benign and malignant breast lesions which typically occur in cohorts of women sent to MRI for breast evaluation. We used these cases to train two pathologists in identifying and quantifying infiltrating immune cells in these breast lesions. Furthermore, we successfully established a panel of antibodies for conventional immunohistochemistry allowing us to decorate different types of immune cells. It is now possible to quantify the cellular players in the immunoinfiltrates in detail which was not possible by conventional histology alone.

In a next step, we wanted to understand the complex interplay of different subtypes of lymphocytes and macrophages with the epithelial cells to gain additional information beyond the simple quantification by immunohistochemistry alone. During the first HYPMED funding period, we were able to raise funds from the DFG (Deutsche Forschungsgemeinschaft) for a novel tissue labelling system to use multispectral fluorescent immunohistochemistry (OPAL) allowing us not only quantification of immune cells but also to analyse their intercellular connections. Establishing a combination of antibodies in OPAL technique required numerous tests which are completed now. We are now able to use the OPAL system to evaluate our immunoscore in each selected tumor group. By using digital image analysis this method can be used in a standardized and reproducible manner not only for our test cohort but also for those breast samples which will be removed in the clinical study of work package 3.
In summary, we were able to establish the technical methods we need for our work package 4. The next step is now to apply our immunoscore in our biorepository sample set. Thereafter, correlation with methylation patterns and imaging will be performed.

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