Chapter 12

Summary and General discussion

Over the past decades, major paradigm shifts have occurred in the treatment of breast cancer. The introduction of population screening, new surgical techniques and (neo)adjuvant therapies greatly improved clinical outcome of breast cancer patients. Examples hereof are the introduction of the sentinel node procedure, neo-adjuvant systemic therapy in patients with locally advanced breast cancer aiming for down-staging of the tumor, thus enabling breast conserving surgery, and adjuvant endocrine treatment [1-3]. Even though major advances in the treatment of breast cancer have been made, mortality remains high. In the Netherlands, each year approximately 14000 patients are diagnosed with breast cancer and 3200 deaths occur as a consequence of this disease [4].

Morbidity associated with current therapy regimens should not be underestimated. Apart from the well-known adverse events of chemotherapy, such as nausea, neutropenia and alopecia, anti-HER-2 and endocrine treatment also harbor a vast array of unpleasant side-effects. For example, anti-HER-2 treatment, Herceptin, is associated with increased cardiotoxicity and hormonal treatment is notorious for life threatening adverse events such as pulmonary embolisms and endometrial cancer, but also less severe incidentals such as hot flashes, vaginal dryness, osteoporosis and musculoskeletal adverse events such as arthritis, arthrosis, arthralgia and myalgia [4-7]. Therefore, prescription of (neo) adjuvant systemic treatment should not be done without careful consideration of the tumor- and patient characteristics.

With the increasing pathological knowledge, tumor classification has become more complex over the last years. Currently, prognostication and treatment allocation are still majorly influenced by tumor stage (TNM) [8;9]. Retention thereto leads to under-treatment and over-treatment [10;11]. Therefore, the use of merely TNM tumor stage for treatment allocation in daily medical practice falls short, and needs to be supplemented with additional tumor biomarkers and patients characteristics that can improve current staging and treatment allocation criteria substantially. Predicting the clinical behavior of a tumor and ultimate clinical outcome of a patient through a combination of clinical, pathological, and biological characteristics will lead to well-targeted treatment of individual patients, hereby increasing treatment benefit and limiting unnecessary adverse-events.

In this thesis we contributed to the foundation for the introduction of precision medicine by evaluating prognostic and predictive biomarkers in breast cancer. The ultimate goal is to improve risk stratification and thus treatment benefit in the individual patient.

This thesis is divided into four parts. In ***part I*** we investigated biomarkers related to important hallmarks of cancer, which were able to adequately assess clinical prognosis in breast cancer patients. In ***part II*** we established the importance of predictive biomarkers, such as human epidermal growth factor receptor-2 (HER-2) and insulin growth factor

1-receptor (IGF-1R), in order to predict who could benefit from directed treatment after breast cancer diagnosis. The potential implication of these (neo)adjuvant therapies on breast cancer in the older is also discussed. In ***part III*** the effect of aging, the most potent risk-factor for oncogenesis, and the entailed metabolic reprogramming, are studied in both healthy and cancer tissue and correlated with the clinical characteristics and clinical outcome of the patients. Finally, in ***part IV*** we discussed the use of prognostic and predictive biomarkers in clinical practice, its utility and the road to precision medicine. Lastly, in ***part V*** the future perspective is discussed.

PART I. PROGNOSTIC BIOMARKERS IN BREAST CANCER

In 2000, Hanahan and Weinberg published an important paper titled: 'the hallmarks of cancer', which initially were six biological capabilities a cell needs to acquire during the multistep developmental process to become a cell with malignant characteristics, ultimately resulting in a full-blown tumor cell. The hallmarks Hanahan and Weinberg proposed are: 1. Sustaining proliferative signaling, 2. Evading growth suppressors, 3. Activating invasion and metastasis, 4. Enabling replicative immortality, 5. Induction of angiogenesis, and 6. Resisting cell death [12]. Eleven years later, in 2011, reprogramming of energy metabolism and evasion of immune recognition were added to the already existing hallmarks [13]. With the addition of the latter two hallmarks, the importance of the tumor-microenvironment in tumor development was taken into account. These cancer hallmarks constitute an organizing principle for rationalizing the complexity of neoplastic disease. Validation and recognition of these much discussed cancer hallmarks will increasingly effect prognostication and effect new means to tackle human cancer [12]. In the first part of this thesis we investigated biomarkers related to these cancer hallmarks, such as sustained proliferative signaling, apoptotic resistance and evasion of immune recognition.

In **chapter 2**, we performed a combined analysis of a proliferative biomarker, Ki67 and apoptotic biomarkers *p*53 and cleaved caspase-3. The inability to undergo apoptosis and the presence of continued proliferation are thought to contribute to tumorigenesis and tumor progression [14-16]. Over the course of years, research performed in this field often showed contradictory results [17;18]. We believe that these contradictory results could be attributed to the fact that a key factor in tissue homeostasis is the balance between the level of cell proliferation and cell death, and that disturbance of this balance could contribute to initiation and maintenance of oncogenesis and tumor growth [12;13]. In our study we circumvented the shortcoming of previous studies by combining the dual markers and constructing an apoptotic-proliferative subtype model. Our study showed

that patients with a high apoptotic marker rate, cleaved-caspase-3, counterintuitively showed worse clinical outcome. However, in the combined analyses, high apoptosis was significantly associated with worse outcome in the presence of a high proliferation rate, indicating that the high proliferation rate outplays the high apoptotic rate, ultimately leading to worse clinical outcome. These data stress the importance of combined analyses for such finely balanced markers, both in immunohistochemical as well as biochemical assays, as is indicated in our study. Furthermore, our study showed that the combination of the two apoptotic (cleaved-caspase-3 and *p*53 status) and proliferative marker (Ki67) into an apoptotic-proliferative subtype model, was also significantly associated with clinical outcome in 488 stage I-III breast cancer patients with respect to overall survival and relapse-free-period. Patients with high proliferation and cellular apoptosis and a mutated *p*53 status had the worst survival and relapse rate outcome. However, only in stage I breast tumor patients this clinical association remained statistically significant in the adjusted analyses. This observation leads to assume that the apoptotic-proliferative subtype model could be of crucial importance in identifying patients with a low tumor grade with an increased risk of poor prognosis, being those containing the most detrimental apoptotic-proliferative marker combination. With the current tendency of earlier breast cancer diagnosis, partly due to better breast cancer awareness and the introduction of population based screening, a shift is seen in the advantage of more early stage detection [19]. Clinical introduction of the apoptotic-proliferative tumor subtype model could lead to targeted selection of the grade I breast cancer patients that would truly benefit of an aggressive therapeutic regime due to an adverse apoptotic-proliferative balance. Especially in current medical practice, which hosts considerable debate on under and overtreatment, identification of patients for the implementation of targeted therapy will continue to conquer ground. Therefore, further research is needed to elucidate the importance of these two hallmarks in light of today's breast cancer related therapeutic standards.

The last decades, research has proposed a substantial influence of the immune system on tumor growth, which showed to be both tumor suppressing and promoting [20]. In **chapter 3,4 and 5** we investigated the prognostic value of important immune recognition evading mechanisms in breast cancer by analyzing classical HLA class I expression on tumor cells; tumor expression of non-classical HLA class I; HLA-E and HLA-G; cytotoxic T-cell tumor infiltration; natural killer cells (NK cells) tumor infiltration and infiltration of immunosuppressive regulatory T cells (Tregs) in the tumor. The goal of these studies was to determine a tumor immune profile based on biomarkers reflecting a tumor's immune susceptibility status and to correlate this with the clinical outcome of each patient.

Previous research has briefly touched on the importance and the complexity of the interaction between breast tumor cells and cells from the immune system [21]. Evidence is accumulating stating that such interactions should be accounted for; therefore we defined tumor immune subtypes, based on tumor expression of immunogenic and immune evasive cellular immune markers.

For 293 breast cancer patients in the training cohort and 219 breast cancer patients in the validation cohort (**chapter 3**), a significant association was found with relapse-free-period and relative survival. Both, relapse rate and relative survival showed worse outcomes in the low immune susceptible tumor types, compared to intermediate and high tumor immune susceptibility. High tumor immune susceptibility was characterized by cytotoxic T-cells being able to recognize tumor-associated antigens presented by classical HLA class I and absence of Tregs (presence of HLA class I, CD8+ cytotoxic T-cells, without Tregs) or, in case of lack of classical HLA class I expression on the tumor surface, resulting in escape of cytotoxic T-cell recognition, natural killer cells come into play and recognize and destroy the diseased cells (loss of classical HLA class I, no expression of HLA-EG, present infiltration of NK cells without infiltration of Treg). Intermediate immune recognition is identified by classical HLA class I expression in the tumor surface, with a lack of cytotoxic T-cell presence or the abundant presence of immune suppressive Tregs, resulting in limited anti-tumor immune reaction. Finally, low immune susceptibility is characterized by the lack of classical HLA class I expression on the tumor cell surface, either in combination with lack of natural killer cells presence, or Treg presence, which results in diminished natural killer cell recognition due to its immune suppressive effect, or lastly, HLA-E and HLA-G presence, resulting in diminished natural killer cell ability of tumor attack. In summary, this study showed a complex and multifaceted interplay between immune cells and tumor cells, resulting in different immune escape mechanisms, highlighting the need for combined immune marker analysis to better reflect patient outcome. In this study we were able to determine three distinct survival patterns in breast cancer based on immune surveillance and escape, which represented significant independent clinical prognostic value in breast cancer patients. Furthermore, evidence is emerging that treatment response is in part regulated by the tumor immune microenvironment [22]. If this holds true, the value of comprehensive determination of the tumor immune status is unthinkably important. Future research should therefore focus on the association between tumor immune susceptibility, preferably taking into account the interplay between immune surveillance and escape, and treatment response.

In **chapter 4**, we investigated the prognostic relevance of the same immune markers as in chapter 3, and the molecular intrinsic breast tumor subtypes in invasive ductal carcinomas and invasive lobular carcinomas separately.

Research has consistently shown that compared to invasive ductal carcinomas, invasive lobular breast tumor tend to have a single-file growth pattern, are larger, more often hormone receptor positive, and harbor a less aggressive character [23;24]. Nevertheless, these two types of breast cancer are still treated similarly, which is largely driven by known classical tumor characteristics such as tumor size, histological grade, hormone receptor status and HER2 status.

Gene expression studies have identified at least four distinct molecular breast cancer subtypes with marked differences in patient prognosis: Luminal A and B, basal-like tumors and tumors overexpressing HER-2. As described above, there is also strong evidence that the breast cancer host's adaptive immune system and the tumors ability to circumvent immune recognition, play a crucial role in the control of tumor growth and progression [25;26]. The aim of this study was therefore to investigate the relevance of the host immune response, the apoptotic-proliferative interaction and molecular tumor types in the two major histological subtypes of breast cancer.

Our results showed no significant difference between invasive ductal and invasive lobular breast tumors with regard to their association with tumor immune subtypes and molecular intrinsic tumor subtypes. Suspicion of the influence of tumor histology on the prognostic value of immune and molecular subtypes was confirmed by a significant effect modifications in the interaction term for immune subtype, the combined cleaved caspase-3- proliferative Ki67 marker and the molecular intrinsic tumor subtypes in relation to relapse rate. In invasive ductal tumors, low tumor immune susceptibility, high cleaved caspase-3 and high proliferative Ki67 expression were associated with a worse relapse free period. This was not seen for invasive lobular tumors, suggesting that neither the apoptotic or proliferative marker, nor immune profiling applies to invasive lobular carcinomas.

Immune profiles were strong prognostic indicators in Luminal A tumors only. This confirms that tumor aggressiveness, as established by the molecular intrinsic subtype of breast cancer, is not dependent on a tumor's immunological profile. Luminal A tumors make up the largest group of invasive ductal breast tumors. Therefore it is not surprising that these results show a similar prognostic association within the immune profiles. Proposed is that invasive lobular tumors harbor characteristics, such as having a high probability of being hormone receptor positive, HER-2 negative, and with a low cellular proliferation rate, making them very probable to be characterized as a Luminal A molecular breast tumor subtype [27]. However, the assumption that therefore molecular and histological subtypes are similar, was not confirmed in our study, implying that a simple extrapolation cannot be made and that breast tumor(s) (subtypes) are presumably far more complex.

Although frequently treated as similar entities, there are obvious differences in tumor-biological and prognostic characteristics for the two major histological subtypes.

Therefore, in order to provide breast cancer patients with the best, targeted treatment it should be stressed that the urgent call for differentiation, especially in therapeutic sense, between these two major histological breast tumor subtypes should be answered. It is of utmost importance that research is performed focusing on the therapeutic sensitivity of these histological breast tumor subtypes, in which, next to classical tumor characteristics, the immune and molecular tumor characteristics should also be accounted for.

In **chapter 5** the difference in prognostic value of tumor immune subtypes in relation with type of hormonal treatment received in hormone receptor positive, postmenopausal breast cancer patients was investigated. Patients of the TEAM trial, consisting of treatment with either exemestane, 25mg daily for five years, or sequential therapy consisting of tamoxifen 20mg daily for 2.5 years followed by exemestane 25mg daily for another 2.5 years [3], allocated in a 1:1 ratio were included in this study. Elaborating on the fact that tumor-associated lymphocytes act as an independent predictor of response to chemotherapy treatment [28;29], evidence also exists for an immunomodulatory effect of the estrogen receptor blocker tamoxifen, inducing a shift from cellular (T-helper 1) to humoral (T-helper 2) immunity [30]. One could speculate on the importance of T-helper 1 immunity for anti-tumor immune response. A shift away from cellular immunity may represent a significant step in tumor development, which could explain the differential effect of aromatase inhibitors versus tamoxifen on clinical outcome [30;31]. Patients assigned to sequential hormonal therapy showed a significant preferential outcome in the adjusted analysis for high FoxP3+ presence in the overall survival. This was not seen for patients in the exemestane only treated arm. This outcome was supported by a significant interaction term for endocrine treatment and FoxP3+ presence in the tumor. This outcome is in stark contrast with the previous studies we performed on tumor immune modulation and cancer development. This result could be explained by the proposition that Tregs harbor a dual role in cancer: being 1. suppressing anti-tumor immune response, known as inducible Treg, and 2. suppressing inflammation which is known to promote carcinogenesis (natural Treg) [32]. It is thought that the clinical and prognostic significance of Tregs in cancer depends on its environmental factors. Given the fact that the TEAM patients are post-menopausal, known for its association with increased systemic inflammation, and are hormone receptor positive [33], herewith attracting higher estrogen levels in and around the tumor due to an increased tendency of estrogen binding, we propose that this milieu leads to more degradation of Adenosine (ADO), a potent anti-inflammatory agent [34;35]. Thus, this line of thought would assume a preference for natural Tregs and would also explain the loss of prognostic significance in solely exemestane treated patients, as aromatase inhibition leads to lower estrogen levels, diminishing ADO degradation. In addition, only for the sequentially endocrine treated TEAM patients the tumor immune subtypes were of significant prognostic value. However, merely a

statistical trend was seen for the interaction between endocrine treatment and tumor immune subtypes in the multivariable interaction model. Given this outcome, one could postulate that the immune profile of breast tumors in sequentially endocrine treated breast cancer patients could predict breast cancer death and overall death in this subset of breast cancer patients, on which additional adjuvant therapy could be allocated.

The result of this study cannot be explained by the previously proposed tamoxifen driven shift from Th1 to Th2 immunity [30]. In that case it would be expected that the difference in prognosis between the high immune susceptible tumor subtype, which is expected to be strongly dependent on cellular Th1 immunity, and the low and intermediate subtypes would be minimized. Reason for this could be that highly immunogenic tumors, by means of other immune interactions, have the ability to circumvent the inferior immune response caused by the tamoxifen-induced Th1 to Th2 shift. Another possibility for the loss of prognostic value of the tumor immune subtypes in exemestane-treated patients could also be Treg dependent. Findings supporting exemestane induced loss of Treg are published previously, proposing a significant increase in the CD8+/Treg ratio in estrogen receptor positive patients responding well to aromatase inhibiting therapy and an observed decrease in FoxP3+ after letrozole treatment [36;37]. One could hypothesize that exemestane induced loss of highly prognostic Treg cells could lead to equalization of the clinical outcomes of the three tumor immune subtypes in the solely exemestane treated adjuvant treatment arm. If this proves true, one could speculate on the great importance of Tregs for inhibition of tumor development in post-menopausal, hormone receptor positive breast cancer patients.

In **chapter 6** of this thesis the prognostic value of the molecular intrinsic breast tumor subtypes in the older breast cancer patient was determined. With four described subtypes, molecular breast tumor classification shows promising prognostic results in modern-day molecular diagnostics [38-40]. Luminal A and B, which are mostly hormone receptor positive and express high amounts of genes related to the luminal epithelial cell layer [38-40], possess the most indolent characters. The basal like tumors, which are triple negative tumors combined with expression of genes characteristic of the basal epithelial layer such as cytokeratin 5 and 6; and the ERBB2 tumor subtype, which clusters near the basal-like subtypes, but expresses high HER-2 on the tumor surface, are both characterized by more aggressive phenotypes, leading to unfavorable outcome [39]. It should be noted that, unlike the HER-2 allocation group in chapter 8 of this thesis, all HER-2 2+ expressing tumors in this study were considered HER-2 negative, due to the lack of confirmatory in situ hybridization and the fact that these elderly patients did not show a significant difference in clinical outcome compared to patients harboring breast tumors with HER-2 scores of 0 and 1+ (chapter 8).

It was proposed that the molecular breast cancer subtypes have a different distribution in older breast cancer patients compared to their younger counterparts [41], and that its prognostic distinction is lost. However, given the fact that that study only included 189 breast cancer patients above the age of 65, leading to very limited discriminative power in the statistical analyses, we felt that validating the prognostic outcome of this previous study in a larger older breast cancer cohort is a valuable contribution. In our study the molecular intrinsic breast tumor subtypes were of significant prognostic value in the older (≥65years) breast cancer population. In accordance with current knowledge, our results were indicative of a higher relapse rate in the ERBB2 and basal molecular breast tumor subtypes and a poor relative survival for all the molecular breast tumor subtypes compared to the Luminal A subtypes. The distribution of the molecular-intrinsic breast tumor subtypes in this older breast cancer population showed a higher prevalence of the more indolent Luminal A tumor and a relatively low prevalence of the more aggressive molecular tumor subtypes compared to the numbers known for the younger breast cancer population [42]. Thus, the chance of getting a more aggressive molecular tumor subtype decreases with increasing age, which is in accordance with the observation of milder tumor characteristics in the older breast cancer population. Furthermore, our results prove the prognostic value of the more aggressive tumor subtypes in the older breast cancer population, reflected in higher relapse rates and worse relative survival.

This is the first study performed in a large older breast cancer cohort, showing significant prognostic value of the molecular breast tumor subtypes, even after taking the risk of competing mortality into account. Therefore, we support the use of molecular subtyping in the older breast cancer patients for prognostication and consequently therapy allocation. However, given the increasing age, we stress that the importance of the functional status of the older breast cancer patient, and the individual treatment wish should not be buried under the molecular force of modern day's diagnostics.

PART II. PREDICTIVE BIOMARKERS IN BREAST CANCER AND TARGETED TREATMENT

Signaling via the Insulin-like Growth Factor type 1 Receptor (IGF1R) plays a crucial role in the development of many cancers, including breast cancer [43;44]. It was shown that IGF1R expression is correlated with the expression of the estrogen receptor [45], and that 17β-Estradiol, although to a lesser extent than IGF1, can activate a linear pathway involving the activation of IGF1R, resulting in a boost of the mitogen-activated protein kinase (MAPK) [46;47]. We proposed that patients treated with an aromatase inhibitor could lose this additional tumor growth-stimulating pathway due to complete blockage of estrogen production, independent of IGF1 stimulation. Furthermore, metformin, which

has long been known for lowering plasma insulin and insulin growth factor levels by increasing insulin sensitivity [48], and thus leading to less IGF1R binding, has also been suggested beneficial in breast cancer treatment [49;50]. In **chapter 7** of this thesis, we performed a sub-study analysis in 2.446 Dutch patients of the TEAM cohort, investigating the clinical effect of exemestane and metformin treatment on IGF1R expression of the tumor in a hormone receptor positive breast cancer cohort. Results of this study showed a significant improvement in relapse free survival in patients treated with exemestane harboring breast tumors with high IGF1R expression their surface, compared to sequentially endocrine-treated patients. No association was seen in low IGF1R expressing tumors. Metformin use in addition to endocrine therapy, resulted in further improvement of the relapse free survival and overall survival in patients harboring high IGF1R expressing tumors treated with exemestane. These interesting findings are in contrast with the main results of the TEAM-trial, which showed no difference in OS, BCSS nor DFS for the two treatment arms [3]. There may be several explanations for the observed benefit of exemestane in patients with high IGF1R expression. Evidence is building for the potential of estrogen to, next to binding and activating its classic estrogen receptor, also phosphorylate and activate the IGF1R [47]. Our results lead to speculate that the interaction between the degree of IGF1R expression on the tumor surface and the efficacy of exemestane is mainly induced by suppressed estrogen production, resulting in reduced estrogen-induced activation of IGF1R and thus less activation of the mitogen-stimulating pathway. This theory also supports our finding that patients with high IGF1R expression who were treated with tamoxifen did not experience a clinical benefit, as these patients still have circulating estrogen in their system, which is able to activate the IGF1R, thereby stimulating breast cancer cell growth. The fact that no clinical benefit of exemestane treatment was observed in patients with tumors harboring low IGF1R expression is also in support of our hypothesis, as the effect of estrogen induced growth promoting signaling through IGF1R is too small in these tumors. We propose that the additive therapeutic effect of metformin is induced by direct lowering of the IGF concentration. In patients with high IGF1R expression on their tumor surface, treatment with exemestane and metformin leads to a dual blockage of the ER-IGF1R crosstalk, resulting in better clinical outcome. By stratifying patients according to IGF1R expression of the tumor, which is up-regulated in roughly two-thirds of the postmenopausal breast cancer population and thus widely applicable, it may become possible to identify a subgroup of patients who may benefit of these combined treatments, thereby further individualizing treatment and improving outcomes for particular subgroups within the heterogeneous BC population. Lastly, we feel that these findings may especially be of interest for the older breast cancer population. Since older patients are at increased risk of toxicity of chemotherapy [51], and around 80% of tumors in older patients are hormone receptor-positive, they are frequently treated with endocrine therapy only [52]. As breast

cancer mortality increases with age, which may be explained by both undertreatment and overtreatment [53], new treatment strategies for this group of patients are highly warranted, preferably with a low toxicity profile. In this study it is proposed that the effect of exemestane may be enhanced by adding metformin without causing additional toxicity, since metformin is a well-tolerated drug with few side effects, thus being a drug with immense potential in the treatment of older patients with breast tumors expressing high IGF1R on their surface.

In **chapter 8** we investigated the potential restoration of the clinical interest of anti-HER-2 treatment in the older breast cancer patients. It is known that HER-2 overexpression is associated with a more aggressive tumor phenotype [54], resulting in worse clinical outcome [55;56]. Treatment of HER-2 overexpression improves clinical outcome in both node negative and node positive breast cancer disease [57;58]. Aberrant activation of the Phosphatidylinositol 3-kinase (PI3K)/AKT pathway by PIK3CA mutations, which often co-occurs with HER-2, results in tumor growth promotion [59], and diminishes response to HER-2-directed therapies [60;61]. A well-known shortcoming in current clinical research of breast cancer patients is that the majority of the studies elucidating the value of biological tumor markers are mainly performed in the younger breast cancer population, impeding extrapolation of study outcomes to the elderly. Foregoing, in combination with increased incidence of cardiac adverse events due to anti-HER-2 treatment resulted in fear of the administration of this drug in the older population. However, evidence for the omission of anti-HER-2 therapy in this specific subset of breast cancer patients is lacking. Therefore, we believe that research is needed to confirm or refute this non-evidence based clinical practice.

This study showed, in 1698 breast cancer patients of 65 years or older (FOCUS cohort), that patients with a HER-2 score of 3+ had a significantly higher risk of recurrence at 5-years post-diagnosis and a worse 10-year relative survival, compared HER-2 negative patients, even when competing risk of mortality was taken into account. Interestingly, patients with HER-2 2+ tumors had a similar recurrence risk as patients *without* HER-2 overexpression. PIK3CA mutations were not of prognostic value for recurrence risk or relative survival in this specific breast cancer population, neither after stratifying for HER-2 status.

These results imply that older patients with HER-2 3+ tumors might benefit from anti-HER-2 treatment. Recent studies have shown that the often severely dreaded, mainly cardiac related adverse events, are in practice less severe and present with a lower incidence than initially thought [62;63]. In current medical practice, anti-HER-2 therapy is frequently omitted from treatment options in the older breast cancer patients due to these shuddered adverse-events, in combination with an often already limited life expectancy. Given the results of this study, it could be suggested that the fit-older breast cancer

patient, in good cardiac health, should be treated with the same adjuvant-regimen as the younger HER-2 positive breast cancer patients. Older patients with less desirable clinical conditions, or with a strong preference to omit chemotherapy, dual HER-2 blockage could be considered. One of the major characteristics of the older cancer population is the heterogeneity patients of the same chronological age. We believe that, if no clear distinction is made between fit and frail older patients, care tends to fall-short for the fit older population, resulting in unfair survival chances. Future research should point out whether it is possible to establish an effective anti-HER-2 regimen with minimal toxicity for the older breast cancer population. It is for this reason that, in this current study, we investigated the difference in expression of HIF-1α and its associated target genes in normal breast tissue and in breast tumor tissue of both young and old patients, and we hypothesize that HIF-1α and its related target genes will be highly expressed and involved in tumor development and maintenance in the older breast cancer population and less so in their younger counterparts.

PART III. AGING IN THE BREAST CANCER PATIENT

Of all the factors that contribute to cancer, aging is the most potent [64].

The multi-hit or Knudson hypothesis, states that cancer occurs more frequently as we age because time is necessary for genetic mutations to accumulate and push these cells over a certain mutagenic threshold [65]. What this hypothesis fails to explain is why cancer risk is greatly reduced by calorie restriction and physical exercise [66]. During aging, the decline in nuclear nicotinamide adenine dinucleotide (NAD^+) levels, leads to a reduction of Sirtuin 1 (SIRT1) activity in the nucleus, causing Von Hippel-Lindau (VHL) to decline and HIF-1α to be stabilized [67]. This age-induced stabilization of HIF-1α, leads to a so-called pseudo-hypoxic state that disrupts oxidative phosphorylation (OXPHOS), thus initiating a Warburg-like state. The subsequent increase of reactive oxygen species (ROS) may establish an environment for subsequent mutations leading to carcinogenesis, which helps to explain why cancer risk increases exponentially as we age [67;68]. The age-induced metabolic decline as a driver of tumorigenesis is also known as "geroncogenesis".

Although hypoxia is toxic for the cell, cancer cells can adapt by genetically modifying oneself to survive, and even proliferate in these stressful conditions. Known cell response to (pseudo) low tissue oxygen levels is through up-regulation of hypoxia-inducible factor-1 (HIF-1). In the (pseudo) absence of oxygen, HIF-1 binds to hypoxia-response elements (HREs), which activate the expression of numerous hypoxia response genes [69]. Known HIF-1 target genes are involved in cell proliferation, angiogenesis, inflammation, metabolism, apoptosis, immortalization, and migration [69;70]. In **chapter 9** we show an increase of HIF-1α mRNA expression and that of its target genes in breast

tumor compared to the normal breast tissue, however this was only seen in patients of 65 years or older, even though there was no significant difference in the pathological tumor stage, grade and tumor morphology for patients <65 years compared to patients of 65 years of age or older. It was noted that for HIF-1α and its targets, the same trend between normal and breast cancer tissue as that observed in the older patient group was also seen in the younger patients of this cohort, implying that HIF-1α and its targets undoubtedly play a role in tumor development of the younger breast cancer patients, but is less stringent then in patients above the age of 65 years. An explanation could be that healthy cells from an older patient are already primed with high HIF-1α expression due to the so-called age-induced HIF-1α stabilized pseudo-hypoxic state, as proposed by Gomes *et al.* [67]. Tumor development, known for its high HIF-1α expression [71], in an already HIF-1α primed environment, results in an exponential increase of HIF-1α in the tumor. Proposed is that the significantly higher HIF-1α expression in the breast tumor of the older breast cancer patients, plays an important role in the more aggressive, and less therapy sensitive character of breast cancer in the old [53]. Therapeutic blockage of HIF, by means of antisense HIF-1α [72], or up-regulation of the VHL gene [73], would result in a reduction of tumor growth, due to a disruption in neovascularization and metabolic reprogramming, which could lead to better clinical outcome. If proven successful, this very promising novel pharmacologic approach to cancer will, based on the expression profiles presented in our study, be of special interest for the older breast cancer patients.

The above-mentioned metabolic shift away from oxidative phosphorylation towards aerobic glycolysis is partly achieved and dependent on the glycolytic enzyme pyruvate kinase(PK) [74]. Normal cells express the pyruvate kinase M1 isoform (PKM1), tumor cells predominantly express the M2 isoform (PKM2). The latter catalyzes the last step of glycolysis and reprograms the glycolytic flux to feed the special metabolic demands of proliferating cells [74]. Over the last decades, PKM2 has identified itself as a promising therapeutic target for cancer treatment, but could potentially also contribute to anti-aging interventions.

In **chapter 10** we investigated the difference in expression of HIF-1α and its associated target genes, including PKM1 and 2, for patients between the ages of 65 and 80 years of age and older (≥80 years) patients in both normal breast tissue and in breast tumor tissue. Next, we investigated whether the degree of expression, or metabolic reprogramming is associated with clinical characteristics associated with aging and outcome. We showed that HIF1-α is significantly higher expressed in the normal breast tissue of the older patient, and that HIF1-α expression in the normal breast tissue is associated with a higher tumor grade of the adjacent tumor. PKM2 had significant association with functional surrogate markers like polypharmacy and difficulty walking, showing a

higher expression in the normal breast tissue of the older breast cancer population, with a potential negative effect on survival.

These observations strengthen the hypothesis that dysregulation of the HIF1-α metabolic pathway, leading to an increase in ROS, is closely related with the high cancer incidence seen in the older population.

On the other hand, our study also showed that high PKM2 *protein* expression in the breast tumor was associated with a significantly better disease free survival and a trend toward better relapse free period compared to patients with low PKM2 protein expression in their tumor, matching the findings of a previous study, showing that activation of PKM2 altered cancer metabolism *in vitro* and reduced xenograft tumor growth [75]. A possible explanation for this finding is the deficiency of precursors for the synthesis of building blocks, favored by dimeric PKM2, needed in high proliferative cellular states. Activation of PKM2 in the active tetrameric form thus inhibits cell proliferation [75;76], resulting in less cancer development and spread. Some advocate PKM2 activation a promising adjuvant treatment modality. However, presence of PKM2, and its importance in the aging process should not be underestimated and could limit its efficacy.

More research is needed to elucidate the potential contribution of HIF1-α and PKM2 on the aging process and the influence on tumorigenesis. If metabolic changes are indeed important drivers of aging and geroncogenesis, molecules that prevent, halt or reverse metabolic aging may be useful anti-aging and anti-cancer therapies. Promising advances have been made with regard to HIF1-α inhibitors, SIRT activators, and both inhibiting, targeting hnRNPA1, hnRNPA2 and PTB, and activating PKM2 treatment [76-79]. Based on current knowledge, it is highly likely that treatment leading to reversal or halting of aging and age-induced disease will experience a rapid development, with major clinical consequences in the coming years.

PART IV. PRECISION MEDICINE IN THE (OLDER) BREAST CANCER PATIENT

Over the course of years it is proven that the TNM stage of the tumor falls short in clinical practice and needs to be supplemented with additional biomarkers to substantially improve current staging and treatment allocation criteria. A lot of research has been dedicated to the discovery and development of clinical prognostic and predictive biomarkers, in order to improve diagnosis and to allocate optimal treatment modalities, introducing precision medicine in the multimodality treatment of cancer. By definition, precision medicine is a multi-faceted approach to medicine that integrates molecular and clinical research with patient data and clinical outcome, and places the individual patient at the center of all elements. Genomic, epigenomic, patient and environmental data are studied altogether to understand individual disease patterns and to design

preventive, diagnostic, and therapeutic solutions. Over the last decades genomic profiling demonstrated its promising prognostic and predictive value in precision medicine, mainly in terms of systemic therapy. Therefore, it is increasingly used in multidisciplinary consultations for risk-assessment and subsequent treatment planning of the individual cancer patient. The added value of genomic profiling on surgical decision making is discussed in **chapter 11**. Apart from a handful of single-gene mutations, genomic tumor profiling in current clinical practice merely directly impacts surgical decision-making. Present-day, influence of genomic profiling on surgery is only seen in the context of profiling of the tumor biopsy, leading to a possible influence on timing, extent and type of surgery by means of optimal tumor shrinkage through targeted neo-adjuvant therapy. Possibly, this may also lead to a wait-and-see approach in case of pathological complete response. This possible influence is not without snags; important questions that need to be resolved are: what is the long-term efficacy of this strategy? Should new follow-up protocols be initiated, and when should the surgical intervention be planned?

To achieve optimum and swift introduction of precision medicine into clinical medical practice, some crucial steps should be warranted:

First, in order to increase clinical applicability, studies investigating biomarkers should focus on using standardized methods and comparable patient selection criteria in order to validate the results. Second, as current cancer research mainly focuses on the genotypical approach of cancer treatment, which is believed to alter cancer treatment radically in the near future, the phenotype of the patient is completely ignored. In the current greying society, it is not uncommon that cancer patients suffer from one or more comorbid conditions, increasing the risk of competing mortality, which therefore should be accounted for when making treatment decisions. Thus, parallel to the current golden standard: TNM stage, and the promising epigenetic and genetic fingerprint of the tumor, phenotypic profiling should not be neglected in the treatment approach of an individual patient. Lastly, medical specialists involved in cancer management need to join forces to create a collaborative multidisciplinary approach, providing the most efficient, functional and tolerated treatment for each cancer patient.

In order to introduce the individualized cancer treatment approach in to daily medical practice, it is required that the medical society is able to overcome these bumps in the road to precision medicine, with as ultimate goal optimal cancer treatment and control.

PART V. FUTURE PERSPECTIVES

The key for appropriate care in the notoriously heterogeneous older breast cancer population lies in the prediction of who will die *with* and who will die *from* breast cancer.

Therefore, the next phase in oncogeriatric research of breast cancer disease is aimed at personalized, tailored treatment.

General treatment decisions in the medical oncology practice are still largely driven by stratified tumor characteristics, such as hormone receptor and HER-2 status. Great efforts are being made to further define tumor characteristics aiming to individualize therapy for breast cancer patients. In order to facilitate this promising shift in treatment modality, future research should emphasize on investigating determinants and markers for tumor response, preferably in the neoadjuvant setting. Under these circumstances, pre- and post-systemic treatment tumor characteristics can be optimally investigated after surgical resection, and correlated with treatment response. Findings of these studies should shed light on what determines whether a tumor will show treatment response or not. These studies would be of enormous value for the older breast cancer patients with regard to the new, preferably minimally toxic treatment modalities, such as angiogenesis blockers, and metabolic stabilizers or reverser, but also for old drugs with potentially new indications, such as metformin.

A well-known shortcoming in today's medical practice and treatment decisions is the disregard of the older (>65 years of age) patients in the clinical trials on which current breast cancer treatment guidelines are based. Consequently, no proven effective guidelines for the older breast cancer population are in operation. It cannot be expected that clinical trials focusing on the older breast cancer patient will void this knowledge gap in the coming years. Therefore, in order to swiftly gain valuable information on the most optimal treatment of the growing older breast cancer patient, in whom, due to lack of feasibility or personal desire chemotherapy and/or surgery are regularly rejected, population based, observational cohorts consisting of older breast cancer patients should be looted of clinical patient and tumor information for research purposes. Expected is that especially the phenotypical, thus functional patient data, not abnegating the competing risk of mortality of each individual patient, will be important treatment drivers which are currently not always adequately accounted for. Thus, in geriatric oncology, it is recommended that treatment decisions are not (solely) based on calendar age. Currently, a lot of research is done in order to determine whether valuable (bio)markers can be identified which could reliably predict ones biological age. The aim of this development is to concise treatment decisions to: adequate anti-tumor treatment, leading to minimal residual disease or adequate supportive care, in order to maintain quality of life.

Only when we abide by the adages: 'treat first what kills first' and 'treat the patient, not the disease', can we achieve the two main treatment goals in the older breast cancer population: 1. prolong survival and 2. maintain acceptable quality of life. With regard to

the first goal; major developments are expected in the coming years, leading to more adequate tumor targets, resulting in optimized systemic therapies. The second goal can only be achieved if the patient's phenotype or functional status is taken into account. For example, frail hormone receptor positive breast cancer patients, with a high chance of dying from other disease, identified by appropriate aging (bio)markers, should preferably be treated with neoadjuvant hormonal therapy instead of neoadjuvant chemotherapy, in which systemic therapy associated toxicities should not be underestimated and if necessary, adequately dealt with.

Only when all medical specialities bound to the care and cure of older cancer patients join forces, better known as a multidisciplinary oncogeriatric battlefront, these treatment goals, and the implementation into daily clinical practice will be achieved.

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