Comparison of primary breast carciomas and multiple corresponding distant metastases

Synopsis of Ph.D. Thesis

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I. INTRODUCTION

Breast cancer (BC) is the most prevalent malignant disease of women worldwide, that affects 1 in 8 women. In Hungary 5-6000 women are diagnosed with the disease every year and nearly 2000 decease because of BC. The 5 year overall survival (OS) of early stage disease (in this case there are no radiologically detectable distant metastases at the time of diagnosis) is over 80%. This high number is the result of organized screening and rapidly developing therapeutic methods (chemotherapy, surgical intervention, radiation therapy, endocrine treatment and other targeted therapies).

BC is a heterogenous disease, with multiple different subtypes with different biological behaviour, prognosis and available therapeutic options. The underlie of this classification are the predictive and prognostic factors studied by immunohistochemistry (IHC) like estrogen receptor (ER), progesteron receptor (PR), human epidermal growth factor receptor 2 (HER2) and the proliferation marker Ki67. Using those markers the subgroups are LuminalA (ER+, PR+, HER2-, low Ki67 index); HER2 negative LuminalB (ER+, HER2-, and PR- or low (≥20%) Ki67 index); HER2 positive LuminalB (ER+, HER2+, any Ki67 and any PR), HER2 positive (ER-, PR-, HER2+, any Ki67) and triple negative, TN (ER-, PR- and HER2- with any Ki67 index). Our group showed previously a pretty high discordance concerning immunophenotype between breast carcinomas and distant metastases, in that study we examined primary tumor - one matched metastasis pairs.

Cancer related mortality is almost always due to metastatic dissemination, around 30% of the patients diagnosed with early BC will develop metastatic disease during their lifetime. In the TN subgroup the risk of developing metastatic disease is the highest in the first 2 years after diagnosis while in case of Luminal tumors it can happen more years or decades after the primary diagnosis. Although as the result of organized screening most of the patients are diagnosed with early BC, there are still some cases being first detected with stage IV or metastatic disease. In these situations the surgical resection of the primary tumor is an often occuring question, that we do not have strong enough evidence about yet. The role of autopsies bacame quite small as we have better and better diagnostic methods to use although these procedures have great potential as we can sample multiple metastatic locations one time and study the course of progression.

While research continues to unravel the molecular underpinnings of the metastatic cascade, it is increasingly recognized that profiling of advanced disease could help elucidate such biological phenomena as distant recurrence and the emergence of de novo resistance to therapy. A handful of studies using genome-wide molecular techniques have begun to explore the clonal relationships between primary and matched metastatic tumours in diverse types of neoplasia including pancreatic, clear-cell renal cell, high-grade serous ovarian and prostate cancer. Despite the small cohort sizes and, too often, a limited number of matched metastases for each patient, these pioneering efforts brought forth thoughtprovoking findings such as the first quantitative model of cancer progression from onset of the founder mutation to metastatic dissemination, the occurrence of organ specific lineages, monoclonal, as well as its counterpart, polyclonal seeding, horizontal cross-seeding between distant metastases and finally homing of metastatic cells to the primary tumour bed. The use of phylogenetic techniques on data generated using whole-exome sequencing studying single nucleotide variants (SNVs) and copy number profiling to identify copy number aberrations (CNAs) can lead us to gain knowledge about the progression and the evolution of the disease. In the past couple of years lots of research focuses on circulating tumor cells and tumor DNA that is a much less invasive way to closely monitor the progression of the disease. While yet other studies continue to highlight the potential of genomic analyses from small cohort sizes to decipher the origins of intra-tumour heterogeneity and its contribution to metastatic dissemination in-depth knowledge is currently lacking for breast cancer. The question is critically important from a clinical and a public health point of view seeing the high number of patients.

II.   AIMS AND OBJECTIVES

1. Characterisation of the primary tumors of metastatic patients; histological type, grade, immunophenotype. Is there a correlation between the immunophenotype of the primary and the localisation of the metastatic lesions?

2. Did the metastatic lesions keep the characteristics of the corresponding primaries?

3. Is there a correlation between the localisation of the metastases and the change in immunophenotype?

4. Are the metastases of the same patient similar to each other (regarding histological characteristics, immunophenotype and genetic parameters)?

5. Is there a correlation between DFS and the change in immunophenotype of the metastases?

6. Monoclonal or polyclonal seeding is more common in the studied patient population?

7. Is there a correlation between OS and the number of genomic alterations in the metastases?

8. Is there horizontal cross-seeding in the any of the studied patients?

9. Is there a clonal relationship between primary tumors and the metachronous contralateral breast tumors?

III.   METHODS

Patients and samples: We reviewed the autopsy database of the 2nd Department of Pathology (2001-2014), Semmelweis University following the permission of the Institutional Review Board (IKEB #185-1/2007). We analysed the parimary and metastatic FFPE samples of 25 patients deceased in metastatic breast cancer. Clinical information was extracted from the patients' medical records.

IHC and FISH: ER, PR, HER2 and Ki67 expression were detected by IHC on all samples using an automated immunostainer system Ventana Benchmark XT. HER2 FISH was performed with Kreatech's Poseidon Repeat-Free probes.

Macrodissection and DNA extraction: DNA was extracted from the primary tumours, metastases and matched normal tissue from FFPE tissue blocks after macrodissection of tumor rich areas using the QIAamp DNA FFPE Tissue Kit. Only 10 patients, whose samples contained >1 μ g of double-stranded DNA, were selected for downstream molecular profiling.

Whole exome sequencing and copy number alterationss: A total of 51 samples including at least one normal sample per patient were sequenced using the Illumina Platform at a target coverage of 40X. The putative somatic SNVs were validated by Sequenom MassARRAY in both the germline reference and cancer samples. As further validation, all available cancer samples were subjected to targeted amplicon deep sequencing at a median coverage of 9,000X to confirm initial sequencing results. For the estimation of CNAs, DNA was genotyped in 64 samples using the Affymetrix OncoScan FFPE Express 2.0 array, samples with <30% cancer cell fraction (CCF) were excluded from the analysis. We used the Dollo parsimony criteria for the best phylogenetic reconstruction using the programme PHYLIP. Integer level estimates of total copy number and major allele were obtained using GAP and MEDICC.

IV.   RESULTS

1. The great majority of the cases (80%) were invasive breast cancers no special type (IBC NST), we detected 2 cases of infiltrating lobular carcinoma (ILC) and pleomorphic ILC (8% both). According to the 25 primary tumor subtypes 8 were TNBC, 1 was HER2 positive subtype, 7 were LuminalA and 9 were categorized as LuminalB: 6 HER2 negative and 3 HER2 positive. Six of 25 tumors were highly proliferating, with a Ki67 score higher than 20%, whereas the mean of Ki67 score was 17.67%. Most of the primary tumors were poorly differentiated, we detected fewer grade 2 and 1 cases (68 vs. 12 vs. 16%).

The most frequent metastatic sites in general were liver, lung, non-axillary LNs and bone. By analyzing the metastatic sites based on the primaries' subtypes, the TNBCs were most likely to metastasize to the liver and lung (88 and 50%, respectively) but did not disseminate to the bones. The LuminalA subtype tumors progressed mostly to the liver and lung (71 and 57%, respectively), but bone metastases were also frequent (43%). Liver metastases were prevalent in the LuminalB group as well (78%). All primaries that metastasized to the skeletal system were HR positive. HR positive tumors metastasized to multiple organs in the body, whereas TNBCs characteristically invaded 2–3 different organs only (mostly liver and lung). The 16 HR positive tumors had distant metastases in 69, the 8 TNBC cancers had metastases in 18 different locations (Fig. 1.)

2. All the TN primaries had TN metastatic lesions. The most frequently seen change of subtype from LuminalA primary to LuminalB or TN metastases as the result of HR loss. LuminalB primary breast tumors were more likely to preserve the immunophenotype in the corresponding metastases, although in half of the cases one or more metastases were TN. Only one of our patient had a local recurrence that was TN subtype just like the corresponding primary. In 12/25 cases the distant recurrences retained the immunophenotype of the primary tumor, there was no loss or gain of receptor expression (Figure 1). From the 16 patients who had an ER positive primary, 10 (62.5%) lost ER expression in one or all metastases. Out of these 16 patients, 11 expressed PR in primary tumors as well. Compared to the primaries, a significant change of PR status was detected; in 10/11 cases (90.9%) loss of PR was noted in one or all corresponding metastases. We

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had 4 patients with HER2 positive primary disease, 2 of which lost HER2 expression in distant recurrences in this group. Gain of receptor (HR or HER2) was not seen in distant recurrences of TNBCs.
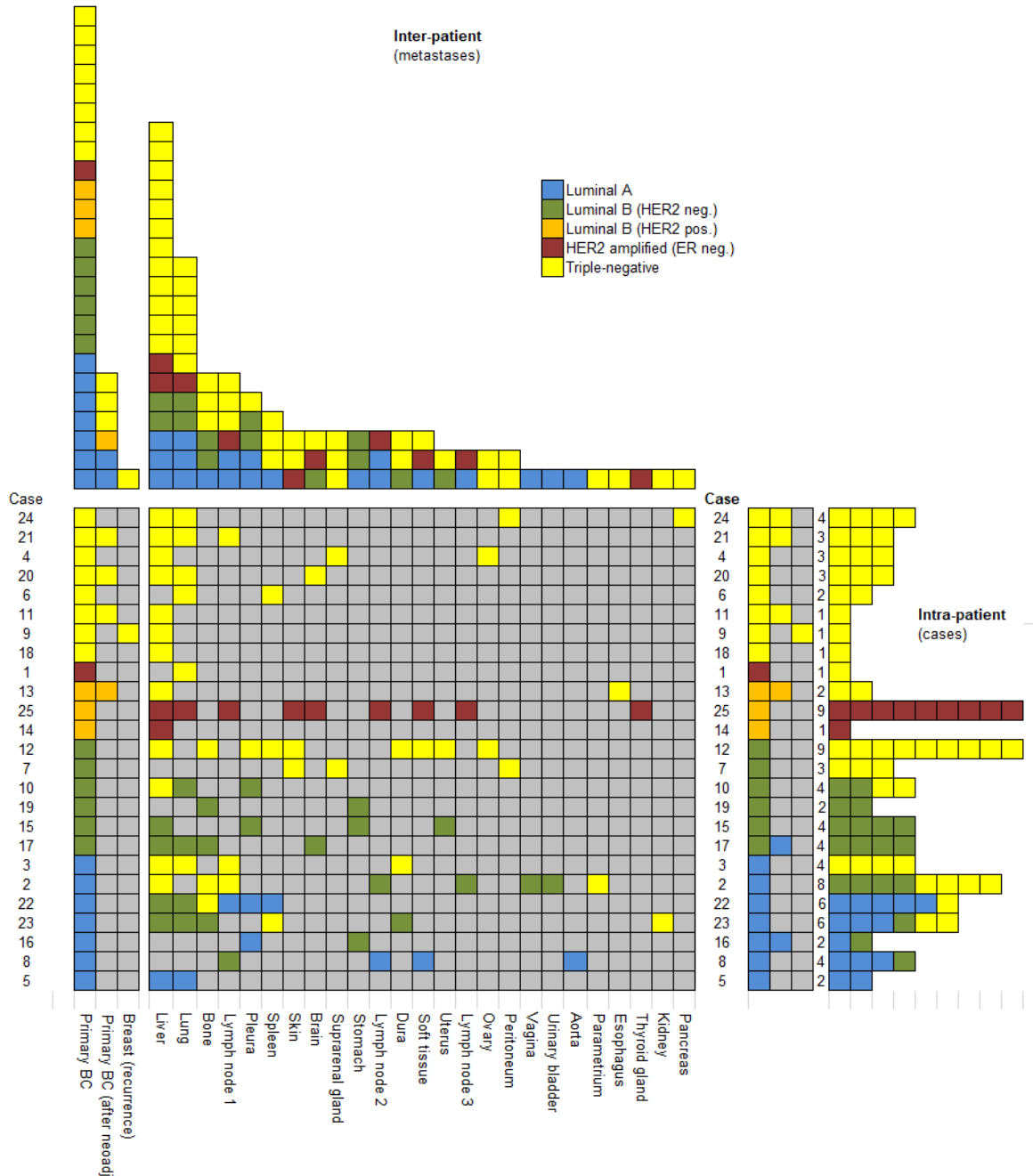
Figure 1. Subtype of primary tumors, local recurrences and distant metastases.

3. The metastases of the same primary tumor were more similar to each other compared to their corresponding primary breast cancer. Based on immunophenotype there were no differences between the groups of metastases compared to each other at any of the steps mentioned above, meaning that the distant recurrences based on localization (regions, organ systems, organs) are very similar to each other. (Table 1.) Metastases were clonally related and originate from cells disseminated at various stages of the disease. Thus, they inherit varying fractions of genomic alterations from their parental lineage, followed by acquisition of private alterations.

4. While analyzing the various metastases in 7/25 cases (in all of them the primary tumor was of luminal subtype) the metastases were heterogeneous; some were discordant while others were concordant with the corresponding primary tumor. Three of 7 patients had the diagnosis of breast cancer at autopsy rendering the clinical history and progression of the disease obscure. In 4/7 patients we were able to follow the course of the disease by retrospectively analyzing the clinical data. In these cases we found a subgroup/time pattern of the metastases: the later the metastasis was discovered the more likely it was to have a differing IHC profile compared to the primary tumor (Fig. 2).
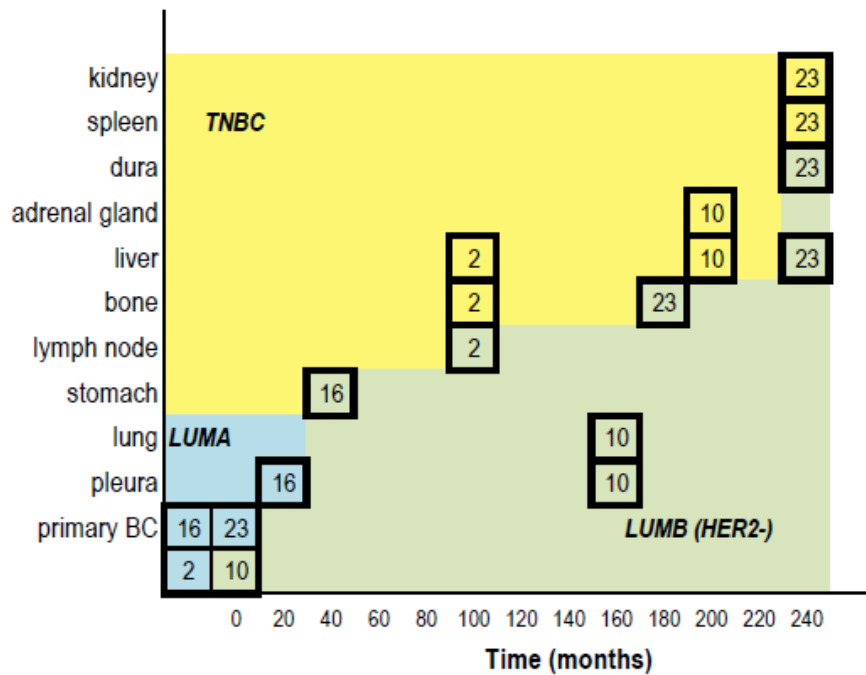
Figure 2. Cases with metastases of heterogeneous subtype.

5. To analyze the distant metastases in more detail we formed a 3-step grouping system based on the localization of the metastases. First we grouped the metastases in five major regional groups: central nervous system (CNS), thorax, abdomen, bone and skin. Second, we divided the metastases to various organ systems, and third, we analyzed the metastases in each organ individually (Table 1).

i. When we studied the diversities between primaries and regions of metastases, we found that tumors were most likely to lose ER expression while metastasizing to the abdomen. Loss of PR expression was detected in thoracic and abdominal metastases also.

ii. Regarding organ systems, loss of ER and PR expression was detected in metastases of the gastrointestinal system. In the respiratory system only the loss of PR expression was significant.

iii. While comparing the primary tumors with their corresponding distant metastases by each organ individually, we observed that the primary tumors were likely to lose HR expression in the liver and lung. Statistically significant change in Ki67 expression was seen only in bone metastases; HER2 status remained unchanged in the above mentioned locations.

Table 1. Difference of ER, PR and Ki67 status in the regions, organ systems and organs compared to the primary tumor.

	ER			PR			Ki67		
PRIMARY	Region	Organ system	Organ	Region	Organ system	Organ	Region	Organ system	Organ
	CNS p=0.268	Dura		CNS p=0.075	Dura		CNS p=0.631	Dura	
		Brain			Brain			Brain	
	Thorax p=0.190	Respiratory system p=0.304	Lung p=0.227	Thorax p=0.039	Respiratory system p=0.05	Lung p=0.049	Thorax p=0.345	Respiratory system p=0.372	Lung p=0.251
			Pleura p=0.829			Pleura p=0.829			Pleura p=0.933
		Others	Aorta wall		Others	Aorta wall		Others	Aorta wall
			Oesophagus			Oesophagus			Oesophagus
			Thyroid gland			Thyroid gland			Thyroid gland
			Soft tissue			Soft tissue			Soft tissue
		Distal LN			Distal LN			Distal LN	
	Abdomen p=0.002	Gastrointestinal p=0.026	Liver p=0.026	Abdomen p=0.000	Gastrointestinal p=0.001	Liver p=0.004	Abdomen p=0.216	Gastrointestinal p=0.213	Liver p=0.212
						Pancreas			Pancreas
						Stomach			Stomach
		Gynecological p=0.123	Ovarium		Gynecological p=0.054	Ovarium		Gynecological p=0.295	Ovarium
			Uterus			Uterus			Uterus
			Vagina			Vagina			Vagina
			Parametrium			Parametrium			Parametrium
		Urological p=0.095	Adrenal gland		Urological p=0.142	Adrenal gland		Urological p=0.322	Adrenal gland
			Kidney			Kidney			Kidney
			Bladder			Bladder			Bladder
		Peritoneum			Peritoneum			Peritoneum	
		Distal LN			Distal LN			Distal LN	
		Spleen			Spleen			Spleen	
	Bone p=0.281			Bone p=0.054			Bone p=0.048		
	Skin			Skin			Skin		

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6. The phylogenies of patients with early BC confirmed that distant metastases probably arose via a seeding event to an initial 'metastatic precursor' from the primary tumour and in absence of the latter, removed at surgery, the source of further dissemination to additional organs occurred by metastasis-to-metastasis disseminations. Our observation suggests that for breast cancer patients diagnosed at an early stage and undergoing curative intent surgery, who represent the majority of patients, cascading disseminations from metastases appears to be a major route of tumor progression (Figure 3a). A contrasting clinical and biological condition to the dissemination via a 'metastatic precursor' is illustrated by the case of patients diagnosed with stage IV. disease, here we identified independent seeding events (Figure 3b).
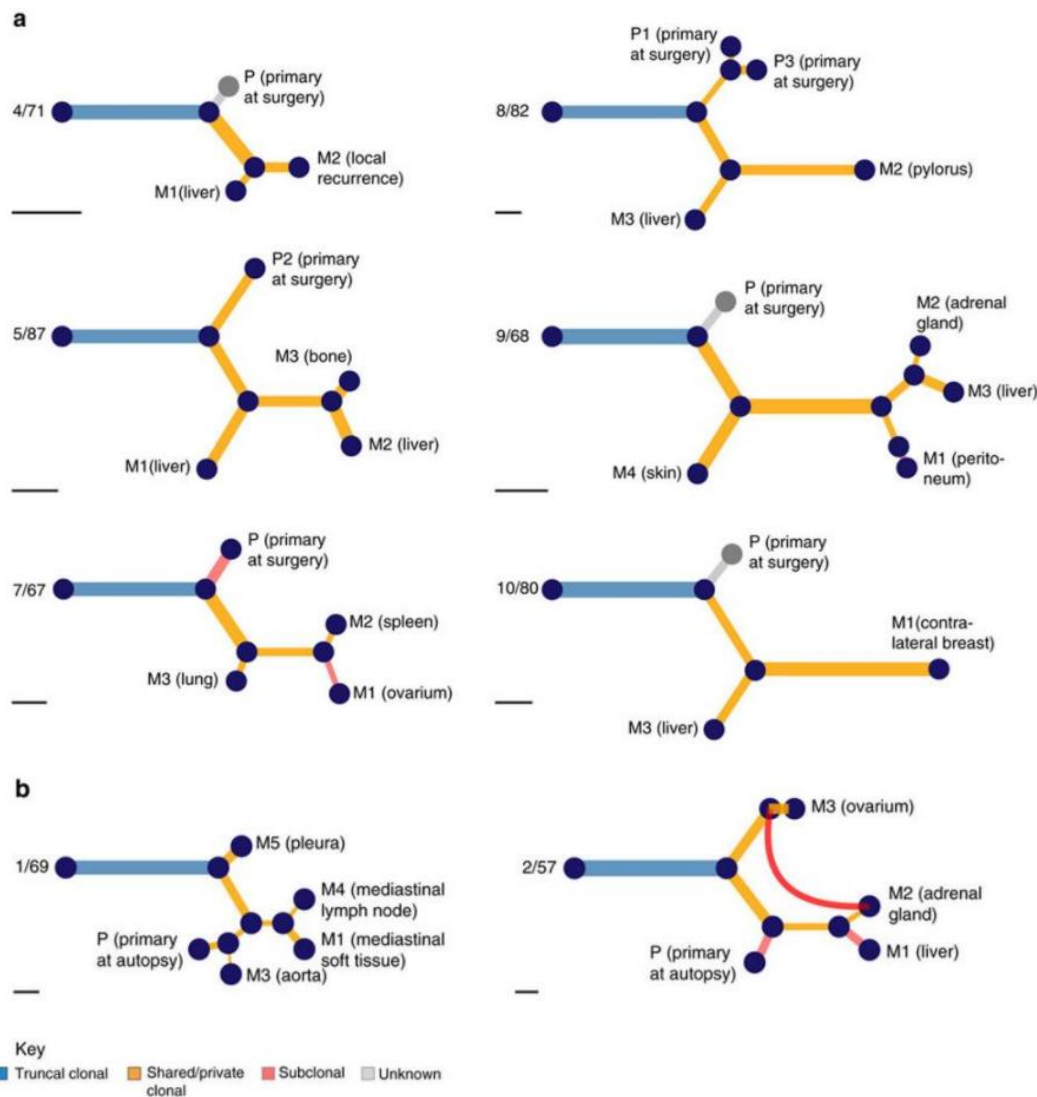
Figure 3. Combined phylogenies representing metastatic progression across eight patients.

7. The normalized phylogenetic branch length, which is the ratio of the path from the common ancestor to the given lesion relative to the common trunk, represents the extent of genomic alterations that accumulated since the first metastasizing event took place irrespective of the mode of progression. If this distance is short, it means that the bulk of evolutionary changes occurred 'early' in the trunk of the phylogenetic tree, in these cases we saw a shorter OS. Figure 4. shows the correlation of the average normalized phylogenetic branch lengths with overall survival. Although the number of patients is small, we observed a positive correlation for both CNAs and SNVs.
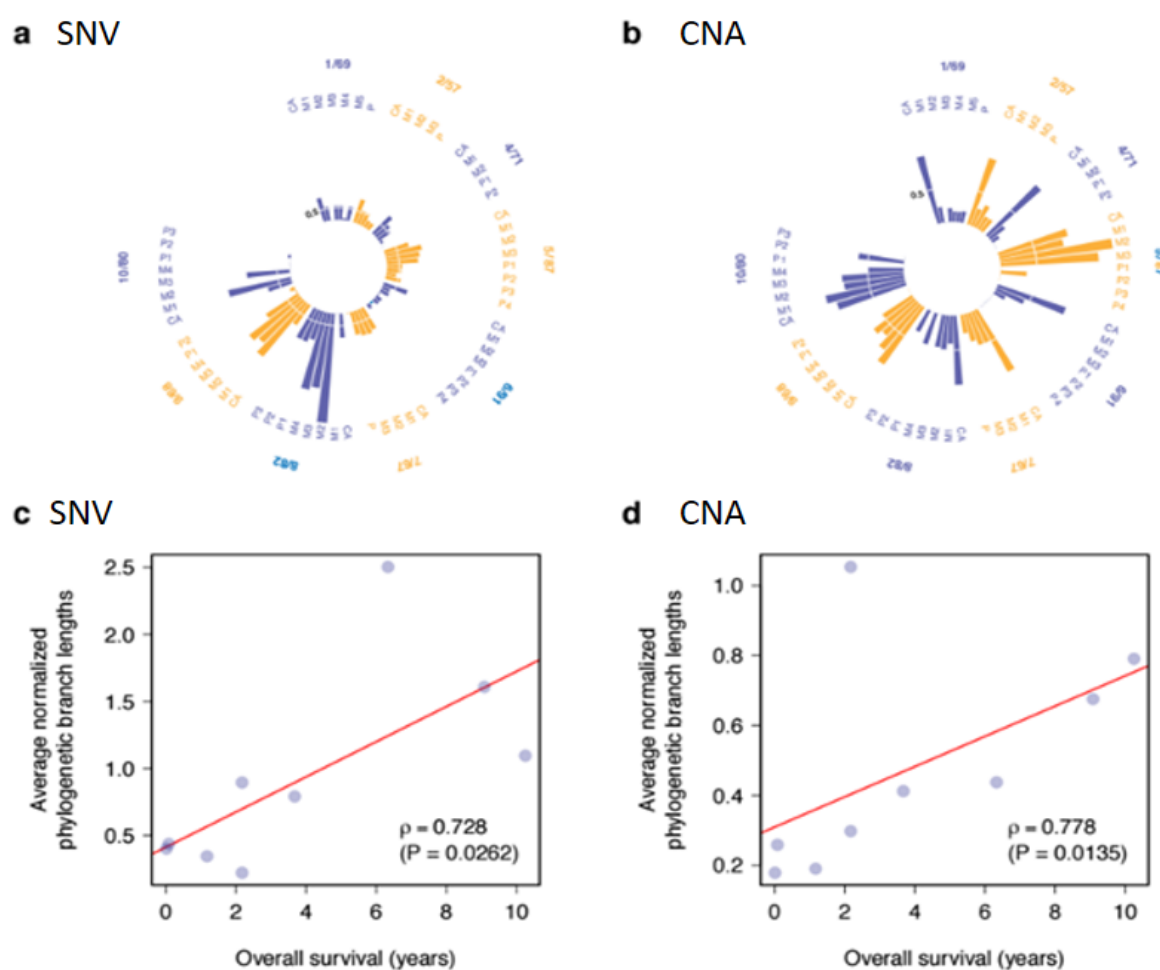
Figure 4. Dynamics of genomic alterations during metastatic progression.

8. In one of our patients we identified two independent seeding events; the phylogenetic analysis of the metastasis to the adrenal gland (M2) revealed that this lesion originated

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from a precursor shared with the liver metastasis. However, the adrenal gland lesion displayed both SNVs acquired 'late' in the evolutionary history of the clade composed of the primary tumour and liver metastasis as well as SNVs private to the ovarian metastasis. Pairwise comparisons of the SNVs showed that those private to the primary tumor and liver metastasis clade were also present at full clonal frequencies in the adrenal gland metastasis in agreement with the phylogeny inferred from the CNA profiles. The 'late' SNVs private to the ovarian metastasis were observed at subclonal frequencies in the adrenal gland metastasis. Our results imply that circulating metastatic cells, disseminated by the ovarian metastasis, horizontally cross-seeded the already metastatic adrenal gland. (Figure 5.)
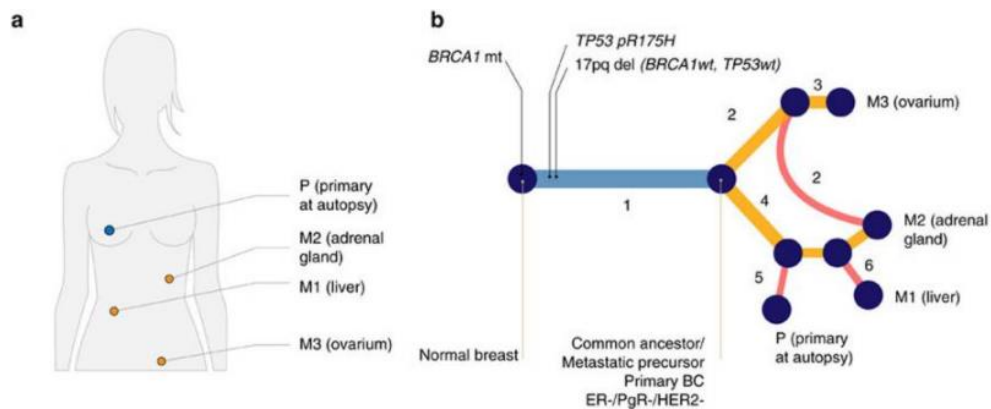
Figure 5. Horizontal reseeding from metastasis to metastasis

9. Two patients from our series were diagnosed with a metachronous contralateral breast tumour. Patient 20, 1 year and Pt14, 10 years after initial diagnosis. In patient 20, the phylogenetic reconstruction showed that the contralateral left breast tumour (M3) was the earliest branching of the tree. In the case of patient 14 the contralateral left tumour (M1) originated from a daughter lesion shared with the liver metastases (M2 and M3). Both cases confirm the clonal relatedness of the contralateral tumour with the initially diagnosed breast cancer. (Figure 6.)
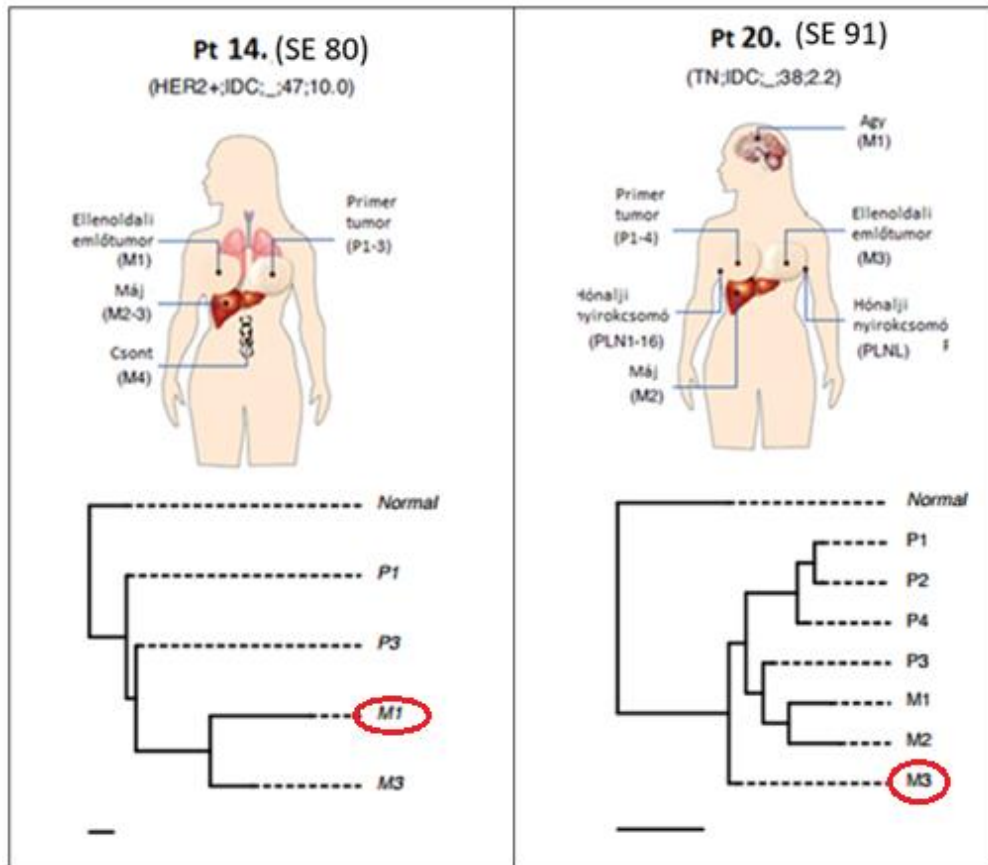
Figure 6. Metachronous contralateral breast tumors as metastatic deposits.

V.   CONCLUSIONS

The aim of our study was to gain a better understanding of breast cancer progression by examining primary tumors and multiple corresponding distant metastases.

Based on our results the metastases of the same primary were more similar to each other and more often differed from the primary lesion. On protein expression level the most common change was the loss of PR, less frequent the loss of ER. It is well known that ER+ PR- tumors have a worse prognosis compared to ER+ PR+ lesions while TN breast tumors have the least favorable prognosis. Based on that the change from Luminal A to Luminal B to TN is a logical way of progression, that might happen in one patient too as the result of disease progression. We detected significant loss of HR in the liver and lung metastases but it is important to notice that these were the locations where we had the highest number of samples.

We observed that the HR positive tumors metastasized to more organs than TNBCs, the usually longer disease history might be a reason behind this phenomenon. The broader metastatic spectrum with more unusual locations is a known feature of HR positive breast cancers.

We had patients with heterogeneous metastases at HR level. Although this occurred only in a minority of the patients, this may be important, since the result of a biopsy from a metastatic site has a crucial role in making decisions about the next line of therapy. Based on our results the longer period of time elapsed between the diagnosis of the primary disease and the appearance of the metastasis, the more likely it was that the metastasis differed from the corresponding primary tumor.

We also applied phylogenetic techniques to infer the evolutionary history of breast cancer progression, compared to previous studies the availability of a larger number of patients with matched primary and multiple metastatic samples was critical to our study for deciphering the routes of dissemination underlying metastatic progression. We observed two possible scenarios. The most frequent implied a single successful seeding event from the primary tumour followed by metastasis-to-metastasis cascading disseminations, whereas the second involved multiple seeding events from the primary tumour alongside daughter metastasis- to metastasis disseminations. This dichotomy coincides with the clinical history where descent from a common metastatic origin was observed in patients diagnosed with early stage breast cancer, whereas multiple seeding events from the

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primary tumour occurred in patients diagnosed with advanced stage disease. The role of primary tumour resection in de novo metastatic breast cancer patients is unclear, and there is currently no consensus whether this procedure confers a survival benefit. Previous clinical trials observed an increased progression free survival for primary tumour resection in HER2- de novo metastatic patients with solitary bone metastases. Thus, our observations suggest that surgical excision of the primary tumour might reduce metastatic dissemination in selected cases hence providing a potential biological rationale for this practice. Similarly, there is no strong recommendation showing overall survival benefit from surgical resection of oligo-metastases in breast cancer. From our analyses, metastatic lesions constitute an additional source of seeding and heterogeneity in advanced breast cancer.

Our results imply the first time in human breast cancer samples, that circulating metastatic cells, disseminated by a metastasis, horizontally cross-seeded another, already metastatic spot. That confirms the previous observations in ovarian and prostate cancers further lending support to the hypothesis of tumour self-seeding.

Our results together with recent reports call into question the current practice of considering metachronous contralateral tumours as second primary cancers. Since treatment strategies offered to patients differ widely between early and advanced stage breast cancers, it is imperative to determine in practice whether contralateral tumours represent a metastatic deposit of the primary tumour.

Our results have some immediate consequences to the clinical practice in treating breast cancer patients, it is important:

      i.   to sample metastatic lesions (at the diagnosis of stage IV. disease, after longer progression free intervals),

      ii.   to determine whether contralateral tumours represent a metastatic deposit of the primary tumour,

      iii.   to resect the primary lesion when the patient is diagnosed with stage IV. in a well defined group of patients.

VI.   PUBLICATIONS

PUBLICATIONS RELATED TO THE DISSERTATION

1. **Szekely B**, Nagy Z I, Farago Z, Kiss O, Lotz G, Kovacs A, Udvarhelyi N, Dank M, Szentmartoni Gy, Baranyai Z, Tokes AM, Szasz AM, Kulka J (2017). Comparison of immunophenotypes of primary breast carcinomas and corresponding multiple distant metastases – an autopsy study of 25 patients.
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