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The role of autophagy in HER2-targeted therapy

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Summary

Macroautophagy (hereafter referred to as autophagy) is a highly conserved, intracellular degradation process characterised by *de novo* formation of autophagosomes. These double membraned organelles engulf and deliver cargo, for example damaged organelles and protein aggregates, to lysosomes for degradation and recycling. Autophagy is primarily a stress response mechanism activated to survive unfavourable conditions such as starvation or hypoxia. In addition, autophagy functions in differentiation, immune responses against invading microorganisms and tissue remodelling in mammalian cells. Besides its cytoprotective nature, and depending on the context, autophagy can as well support cell death. Based on autophagy's cytoprotective, cytotoxic and developmental influences, it does not come as a surprise that this mechanism is involved in tumourigenesis, tumour development and the response to anticancer therapies. HER2 is a receptor tyrosine kinase that activates downstream signalling pathways involved in cellular survival, growth and proliferation. Amplification of the gene and subsequent overexpression of the HER2 protein lead to increased activation of downstream signalling and are implicated in several cancer types. HER2-targeted therapies are valuable treatment options for HER2 amplified cancers. However, pre-existing and acquired resistance remain a clinical challenge. Autophagy has been discussed in several scenarios in HER2 amplified cancers. Generally, $HER2^{+}$ tumours have been shown to exhibit low levels of proteins essential for autophagy. Moreover, a protein involved in autophagy activation, Beclin-1, was shown to interact directly with HER2 at the cellular membrane. The signalling cascade activated by HER2 also activates mTOR, a negative regulator of autophagy. In the context of resistance formation against HER2-targeting treatment, autophagy has often been reported to be upregulated, and resistance has been shown to be abrogated through autophagy inhibition. Since the autophagy inhibitors chloroquine and hydroxychloroquine are approved drugs for the treatment of malaria, autophagy inhibition is discussed as an option to enhance the effect of certain anticancer treatments or to overcome resistance against cancer therapies. In this review we focus on autophagy and its role in the response to HER2-targeted therapies for breast and gastrointestinal tumours.

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Autophagy

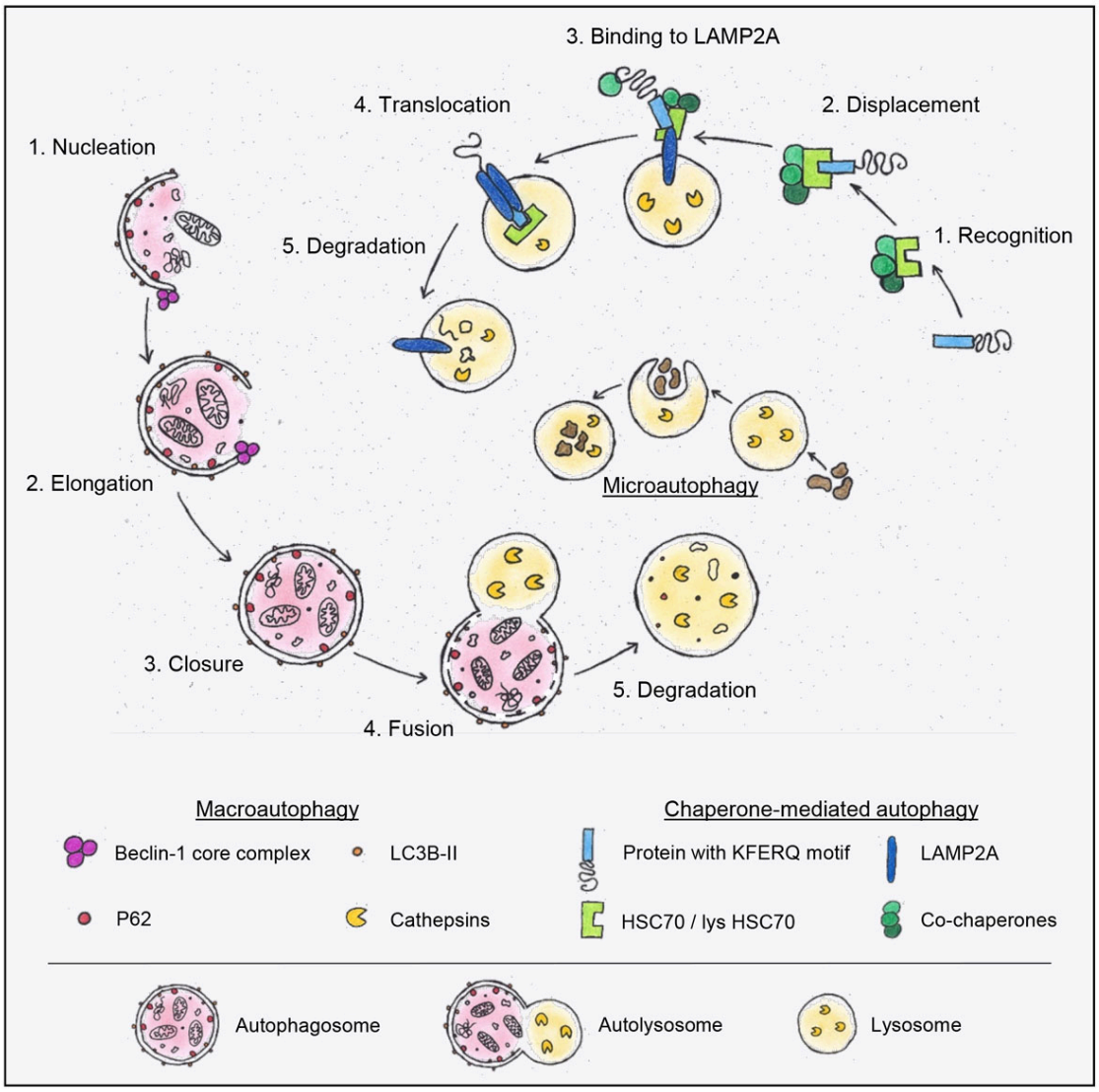
Macroautophagy, often simply referred to as autophagy, is a multistep process involved in cellular homeostasis and adaptation to stress conditions. As a catabolic process, autophagy maintains the nutrient homeostasis of the cell and participates in the quality control of proteins and organelles [1]. Upon cellular stress, for instance by starvation, hypoxia, genotoxic or proteotoxic stress, autophagy is upregulated as an adaptive cell response [2, 3]. The process is characterised by the formation of a double membraned vesicle, the autophagosome, which engulfs cytoplasmic material. The autophagosome fuses with the lysosome, which ultimately leads to the degradation of its content. In the lysosome, proteins are degraded by cathepsins, which are a group of proteases activated at low pH values typical of lysosomes [4]. Thus, the content of the so-called autolysosome is recycled for biosynthesis and/or energy production. The macroautophagic pathway is divided into distinct steps (fig. 1): (a) nucleation of the isolation membrane, (b) expansion of the membrane, (c) closure and maturation of the autophagosome, (d) fusion of the autophagosome with the lysosome, and (e) degradation and recycling of the delivered cargo [5, 6]. The evolutionarily conserved degradation pathway involves at least 16–20 core autophagy (ATG) genes. The ATG proteins encoded by these genes are classified into functional groups that act at different stages of autophagy [7, 8].

One of the important players in this multistep process is unc-51-like autophagy activating kinase 1 (ULK1, the mammalian orthologue of yeast ATG1). It is the activating kinase in the autophagy initiating complex [9, 10]. The phosphorylation of downstream players by this complex leads to the elongation of the isolation membrane and allows the recruitment of another multiprotein complex containing Beclin-1 and the catalytic subunit VPS34 [11, 12]. Beclin-1 is monoallelically deleted or downregulated in various tumour types, such as breast and ovarian cancer, indicating its tumour suppressor function [13, 14]. The isolation membrane elongates through incorporation of phospholipids from different sources, such as the endoplasmic reticulum (ER). As autophagy is a dynamic process, it is difficult to capture the actual "autophagic flux" describing the rate of degradation by using only ATG gene expression

data. Therefore, the microtubule-associated protein light chain 3B (LC3B) is frequently used as an autophagy marker. The LC3B protein is processed during active autophagy. As an ATG8 family member, it is lipidated to LC3B-II and then integrated into the growing autophagosomal membrane by its conjugation to phosphatidylethanolamine. LC3B-II enables the elongation of the isolation membrane as well as cargo recruitment [15, 16]. The non-lipidated LC3B-I and the lipidated LC3B-II can be differentiated by Western blotting. Thus, increasing autophagosome formation can be visualised via Western blotting and indicated by higher LC3B-II to LC3B-I ratios. Additional LC3B-based techniques include assessment of LC3B dot formation upon ectopic expression of GFP-LC3B or of endogenous LC3B, using immunofluorescent microscopy or immunohistochemistry [17].

Under certain conditions, such as starvation, autophagy is rather unspecific. In the last decade, however, specific degradation of cargo by macroautophagy, so-called selective autophagy, has been described. Different cargos, such as mitochondria, lipid droplets, ribosomes, protein aggregates or individual proteins can be degraded via selective autophagy with the help of cargo receptors. These cargo adaptor proteins directly link the cargo to the autophagosome. In most cases the cargo either contains a so-called LC3-interacting region (LIR) that can bind to LC3B or the cargo is labelled with a ubiquitin tag. In the latter case, the degradation is mediated by an adapter protein that has a ubiquitin binding site as well as an LIR [18]. Sequesto-

Figure 1: Schematic presentation of macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy. On the left hand side, an overview of macroautophagy is depicted in red. The isolation membrane forms during the nucleation process. An important player in nucleation and elongation is the Beclin-1 core complex. During the elongation of the double membrane LC3B is lipidated and allows the binding of adaptor proteins such as p62. After autophagosome closure, the autophagosome fuses with the lysosome, followed by degradation and recycling of its contents. The degradation is mediated by cathepsins. These proteases become activated in the low pH found in lysosomes. In the middle of the diagram, microautophagy, a process characterised by the direct uptake of cytosolic material into the lysosome, is depicted. On the right, the chaperone mediated autophagy (CMA) pathway is outlined. In this process, proteins containing a KFERQ amino acid sequence motif are recognised by a chaperone complex containing HSC70. The complex is then translocated to the lysosome and, with the help of the LAMP2A complex, incorporated into the lysosome and degraded.p62 = sequestosome 1, SQSTM1; LC3B = microtubule-associated protein light chain 3B; HSC70 = heat shock 70 kDa protein 8; LAMP2A = lysosome-associated membrane protein 2A



some 1 (SQSTM1, also known as p62) represents just such an autophagy adaptor that targets cargo to the growing autophagosomal membrane. P62 is degraded together with the cargo, which allows using degradation of p62 as a marker for autophagic flux [19, 20].

Besides macroautophagy there are at least two additional forms of autophagy, namely chaperone mediated autophagy (CMA) and microautophagy. These three pathways differ in the way the cargo is delivered to the lysosome (fig. 1). During CMA, cytosolic proteins are recognised by heat shock 70 kDa protein 8 (HSC70) through a specific amino acid sequence, the so-called KFERQ motif. Subsequently, the protein is shuttled to the lysosome and is finally translocated via another protein complex including lysosome-associated membrane protein 2A (LAMP2A) into the lysosome (fig. 1) [21, 22]. Microautophagy is characterised by the direct lysosomal uptake of cytoplasmic entities [23].

In addition to its role in homeostasis and stress adaptation, autophagy also plays a role in cellular differentiation, immune response against invading microorganisms or tissue remodelling [24, 25]. Aberrant autophagy has been reported in connection with various diseases, such as inflammation, cancer formation or neurodegeneration [2, 26]. In this context it is important to mention that autophagy and apoptosis are closely linked. The anti-apoptotic protein Bcl-2, which is often upregulated in cancer, directly interacts with the key autophagy gene Beclin-1. By binding and sequestering Beclin-1 and thus inhibiting it from initiating autophagy, Bcl-2 acts as a negative regulator of autophagy [27]. Moreover, additional ATG proteins, such as ATG5 or ATG12, interact with Bcl-2 family members, indicating a complex crosstalk between the two pathways [28, 29].

To summarise, the term autophagy describes several lysosomal degradation pathways that differ in their cargo delivery to the lysosome. The best-studied form of autophagy is macroautophagy. The conserved multistep process is responsible for cellular homeostasis at basal conditions and can be upregulated upon different cellular stresses. In recent years, macroautophagy has been shown not only to perform bulk degradation but also to exert high cargo specificity. The cellular stress response mechanism autophagy is interconnected with apoptosis, and aberrant autophagy has been linked to various diseases.

Autophagy and cancer

Autophagy plays a dual role in cancer development and progression. Autophagy guards cellular homeostasis and therefore contributes to the prevention of malignant transformation. On the other hand, it seems to play a rather tumour-promoting role in established tumours (fig. 2) [30]. Evidence to support a tumour suppressor function of autophagy stems from murine models defective in essential autophagy genes. For instance, *Beclin-1*$^{+/-}$ animals spontaneously develop malignancies such as lymphomas or lung carcinomas [31, 32]. Moreover, mice with liver-specific knockout of *Atg7* or a systemic mosaic deletion of *Atg5* develop benign hepatic neoplasms [33]. Additionally, autophagy suppresses the accumulation of genetic and genomic defects caused by reactive oxygen species (ROS) through removal of dysfunctional mitochondria and redox-active aggregates of ubiquitinated proteins [34, 35]. Moreover, by eliminating dysfunctional mitochondria autophagy ensures optimal energy supply, which counteracts the metabolic rewiring often observed during malignant transformation [35]. Further, autophagy is involved in the maintenance of normal stem cells. The above-mentioned *Beclin-1*$^{+/-}$ mice, for example, show an expansion of progenitor-like mammary epithelial cells [36, 37]. Autophagy is also involved in the degradation of aggregate-prone oncogenes, such as forms of mutated TP53, p62, PML-RARA or BCR-ABL1 [34, 38–40]. It is required in several aspects of anticancer immunosurveillance, and thus in the elimination of potentially tumourigenic cells by the immune system [41]. Additionally, autophagy plays a key role in first line defence against bacterial or viral infection. Multiple potentially carcinogenic pathogens, such as *Salmonella enterica*, *Helicobacter pylori* or *Chlamydia pneumoniae,* can activate autophagy upon infection [42–45].

There are indications that the activation of oncoproteins, and, similarly, the inactivation of tumour suppressor proteins can attenuate autophagy. This reduced autophagic activity supports early phases of oncogenesis [46]. Anti-apoptotic Bcl-2 family members, such as Bcl-2 or Bcl-X_L, that are upregulated in various cancer types, also inhibit autophagy through sequestration of Beclin-1 [27]. MDM2 represents another proto-oncogene that negatively affects autophagy. High MDM2 levels inactivate the TP53 tumour suppressor. Inactivated TP53 then fails to activate transcription of its target ATG genes [47]. Furthermore, several receptor tyrosine kinases (RTKs), such as the epidermal growth factor receptor (EGFR) or v-erb-b2 avian erythroblastic leukaemia viral oncogene homologue 2 (ERBB2, also known as HER2), or downstream signal transducers that are often overexpressed in solid tumours inhibit autophagy by activating its negative regulator mTORC1 [48]. The tumour suppressor phosphatase and tensin homologue (PTEN) is often inactivated in cancers. This phosphatase promotes autophagy by antagonising PI3K signalling that negatively regulates autophagy [49]. The transcription factor forkhead box O1 (FOXO1) represents another tumour suppressor essential for stress-induced autophagy that is mutated in diffuse large B-cell lymphomas [50].

In neoplastic cells, however, restored autophagy response allows cancer cells to cope with intracellular and environmental stress (fig. 2, right panel) [51, 52]. Thus, in advanced human tumours high autophagic flux correlates with an invasive, metastatic phenotype and poor survival rates [53]. In mouse experiments, highly metastatic hepatocellular carcinoma cell lines with inhibited Beclin-1 or Atg5 expression are unable to survive in the metastatic niche, in contrast to their autophagy-competent counterparts [54]. In KRAS-driven pancreatic adenocarcinoma cells, autophagy is upregulated upon oncogene ablation to counterbalance the metabolic stress occurring upon shutdown of oncogenic KRAS signalling [55]. Breast cancer stem cells from mammosphere cultures are also characterised by elevated autophagic flux. Importantly, their ability to form tumours *in vivo* seems to depend on proficient autophagy, as they are not able to form tumours upon genetic inhibition of Beclin-1 or ATG4A [56, 57]. Autophagy-deficient tumours are generally more sensitive to chemotherapeutic agents and to radiotherapy compared with their autophagy-proficient counterparts [58, 59].

Senescent cancer cells, which do not proliferate but can still support relapse by influencing the tumour microenvironment, depend on autophagy for survival [60].

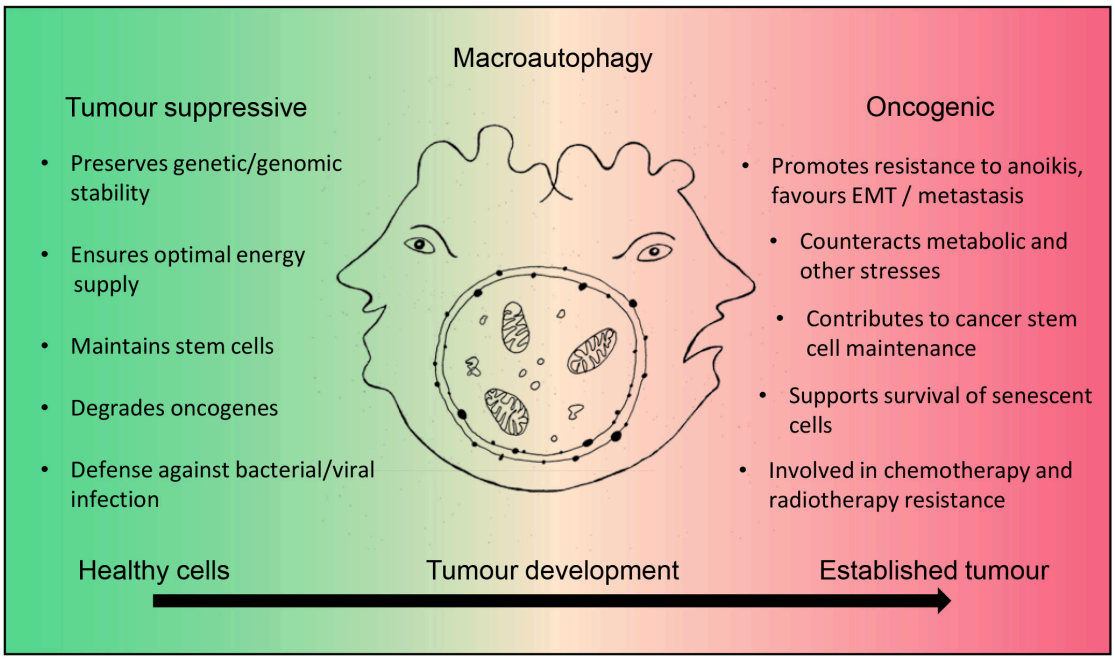
In summary, autophagy in healthy cells prevents tumour development, and its downregulation may contribute to early oncogenesis. In late tumour development cancer cells hijack autophagy to play instead an oncogenic role (fig. 2). In this context, autophagy can protect cancer cells from anticancer treatment and may thus contribute to therapy resistance.

Several compounds that inhibit autophagy at different stages are known. The only clinically approved autophagy inhibitor is the antimalarial drug chloroquine and its derivative hydroxychloroquine. These drugs interfere with lysosomal acidification and thus prevent the degradation of autophagosomes [61, 62]. Hydroxychloroquine is preferred over chloroquine in clinical trials owing to its lower toxicity [63, 64]. Several preclinical *in vitro* and *in vivo* studies have shown an antineoplastic effect of hydroxychloroquine in combination with various clinically approved drugs [65]. However, the use of chloroquine and hydroxychloroquine as autophagy inhibitors is controversial, since both reagents exert autophagy inhibition-independent effects as well. Thus, chloroquine can sensitise cancer cells to chemotherapeutic drugs independent of autophagy inhibition [66, 67]. This was illustrated in a 2012 study, where chloroquine was used to sensitise mouse breast cancer cells to several established anticancer treatments. The observed sensitisation was independent of autophagy inhibition as it could not be mimicked with ATG12 or Beclin-1 knockdown or treatment with Bafilomycin A1 (BafA), another autophagy inhibitor [66]. Furthermore, a preclinical study evaluating the pharmacodynamics of hydroxychloroquine in pet dogs with lymphoma showed that the plasma concentration of hydroxychloroquine does not correlate with drug concentration in the tumours. This discrepancy consequently also applies to the extent of autophagy inhibition in the tumour tissue [68]. Another open question concerning the use of chloroquine and hydroxychloroquine as autophagy inhibitors in the clinic is whether it is better to block the process at early or at late autophagy stages. Early autophagosomal structures can serve as scaffolds for inducing apoptosis and necroptosis. Thus, the accumulation of autophagosomes could promote these pathways in some cases [69]. Since chloroquine and hydroxychloroquine inhibit autophagy at a late stage, and their mechanism of action is not well understood, new specific inhibitors with improved pharmacodynamics that also target early autophagy kinases, such as VPS34 or ULK1, are being developed and evaluated in preclinical studies [70, 71].

Human epidermal growth factor receptor 2 (HER2) and its role in human cancers

HER2 (also known as ERBB2) is a transmembrane receptor tyrosine kinase of the EGFR (epidermal growth factor receptor) family, consisting of EGFR (ERBB1), HER2 (ERBB2), HER3 (ERBB3) and HER4 (ERBB4) [72, 73]. These receptor tyrosine kinases act in the epithelium as signal transducers between mesenchymal and epithelial cells. The EGFR receptors form homo- and heterodimers which transphosphorylate each other upon activation. This further stimulates intracellular downstream pathways, such as mitogen-activated protein kinase (MAPK) signalling

Figure 2: The Janus-faced role of autophagy in cancer. In healthy cells macroautophagy has a tumour suppressor function. As a catabolic survival mechanism, it ensures optimal energy supply, preserves genetic and genomic stability, maintains normal stem cells, is involved in the degradation of oncogenes, and is the first line defence against bacterial or viral infection. During tumour formation downregulation of autophagy is frequently observed, possibly supporting tumor development. In established tumours, however, autophagy functions can be restored and instead support tumour development. This occurs, for example, via (a) supporting EMT and metastasis, (b) rendering cells resistant to anoikis (programmed cell death upon detachment), (c) counteracting metabolic and oxidative stress, (d) maintaining cancer stem cells and supporting the senescent cell state, and (e) promoting chemo- as well as radiotherapy resistance.



cascades RAS/MEK/ERK, PI3K/AKT/TOR or STAT transcription factors, that are involved in proliferation, survival and differentiation [74, 75]. Examples of ligands shed by the mesenchyme are Neuregulins that bind to HER3 and HER4. Although HER2 is the most potent oncogene of the family, to date no high affinity ligand for HER2 has been found. However, its conformation resembles a ligand-activated state, which favours dimerisation [76, 77]. This makes HER2 the preferred dimerisation partner for the other three family members [78]. As the signalling system may be fine-tuned by the partners of the heterodimer, the abundance of HER2 is crucial. Importantly, the HER2-HER3 heterodimer is the most transforming and mitogenic heterodimer of the EGFR family [79, 80]. The potent proliferation signalling generated by the EGFR network is often corrupted in cancer cells. Overexpression or constitutive action of the individual receptors is observed in a variety of tumours [81]. HER2 is overexpressed in several types of tumours such as breast, gastric, oesophageal, lung, bladder or endometrial cancer [82]. However, in breast cancer the association of *ERBB2* amplification and subsequent overexpression of HER2 is the best studied. About 20% of breast tumours show *ERBB2* gene amplification [83]. The amplification correlates with high risk of recurrence and disease-related death and thus a poor prognosis [84, 85]. $HER2^+$ tumours form a separate subclass of breast cancer that is eligible for HER2-targeted treatment. These tumours are typically oestrogen receptor negative. Moreover, HER2 expression is the most important predictive factor for response to HER2-targeted therapies [86]. Similarly, HER2 overexpression correlates with pathological features such as lymphatic invasion, high grade or large tumour size in gastric cancer [87]. Besides the *ERBB2* amplification, somatic mutations are observed in several tumour types, such as breast, lung, gastric and bladder cancer. Most of these mutations are missense mutations in the tyrosine kinase or extracellular domain, rendering the receptor constantly active [88].

HER2-targeted therapies

HER2-targeting drugs to treat $HER2^+$ tumours can be divided into two classes: (a) HER2-directed antibodies, such as trastuzumab, and (b) small-molecule inhibitors targeting the kinase activity of the receptor, such as lapatinib [89]. Trastuzumab is a humanised antibody that binds an extracellular epitope of HER2. Binding of the antibody uncouples HER2-containing dimers, which leads to partial inhibition of downstream signalling. In addition, trastuzumab induces antibody-dependent cell-mediated cytotoxicity (ADCC) [90–92]. As early as 1998, trastuzumab was approved for metastatic breast cancer, and in 2006 approval for adjuvant therapy of early breast cancer followed. Trastuzumab has been successfully integrated into standard therapy for $HER2^+$ breast cancer, either in a perioperative or metastatic setting [93–96]. In 2010, trastuzumab was approved for advanced gastric and gastro-oesophageal cancer. Previously, the ToGa (trastuzumab for gastric cancer) study showed a significant overall survival benefit for patients with metastatic $HER2^+$ gastric cancer when the drug was added to standard chemotherapy [97]. A new generation of HER2 antibody, pertuzumab, recognises a different epitope of HER2. Its binding leads to the blockage of ligand-induced HER2-HER3 dimerisation and thus inhibits partially downstream signalling [98]. As trastuzumab and pertuzumab target different epitopes, the combination of the two antibodies showed a synergistic effect in preclinical studies and clinical trials [99–101]. Since 2012, pertuzumab has been approved for the treatment of $HER2^+$ metastatic breast cancer [102]. Another derivative of trastuzumab is trastuzumab emtansine (T-DM1). This is an antibody-drug conjugate whereby trastuzumab is bound to maytansinoid, a drug inhibiting microtubule polymerisation [103]. This new antibody-drug conjugate binds to the epitope with an affinity similar to trastuzumab, and, in addition to blocking signal transduction and induction of ADCC, the drug mediates the inhibition of microtubules [104]. In 2013, T-DM1 was approved for advanced $HER2^+$ breast cancer [105].

In contrast to trastuzumab, lapatinib is a small-molecule kinase inhibitor that binds reversibly to the ATP-binding side of EGFR and HER2. Lapatinib disables HER2 downstream signalling, being effective even in $HER2^+$ cancers that have progressed after trastuzumab treatment. Lapatinib was approved for the treatment of advanced breast cancer in 2006 [106]. Further developed derivatives are afatinib and neratinib. Both small-molecule inhibitors bind irreversibly to the ATP-binding site of RTKs. Whereas neratinib binds only to HER2, afatinib binds to both HER2 and EGFR [107, 108]. Neratinib was approved for the adjuvant treatment of $HER2^+$ breast cancer after trastuzumab progression in 2017 [109]. Afatinib, as a dual EGFR-HER2 inhibitor, is approved for the treatment of non-small-cell lung carcinoma [110]. Besides the considerable success of HER2-targeting drugs and their improvements, resistance formation remains a clinical challenge.

Resistance mechanisms against HER2 inhibition

Although HER2-targeting treatment provides considerable benefit for patients with $HER2^+$ tumours, most tumours ultimately progress to treatment resistance [111]. Resistance may be pre-existing or drug-induced (acquired). Generally, pre-existing resistance tends to occur through alterations of the receptor itself or modifications of downstream signalling pathways [112, 113]. On the other hand, acquired resistance is more diverse. Here, resistance instead occurs through bypass mechanisms as increased expression of other family members (EGFR, HER3) or different receptor tyrosine kinases, such as hepatocyte growth factor receptor (MET) [114–116]. It is important to mention that all the resistance mechanisms described above can be the cause of pre-existing as well as of acquired resistance [117–119].

Expression of a truncated form of HER2, so-called p95-HER2, which lacks the trastuzumab binding epitope, represents an alteration of the HER2 receptor mediating treatment resistance [113, 120]. P95-HER2 is associated with low response rates to trastuzumab. However, since the kinase activity of the receptor remains intact, these tumours are still sensitive to kinase inhibitors such as lapatinib [121]. Another HER2 alteration that mediates resistance is an *ERBB2* gene splice variant lacking exon 16. These receptors retain the epitopes recognised by trastuzumab, but HER2 homodimers containing this isoform are more stable than their wild-type counterparts.

Therefore, trastuzumab-mediated disruption of the homodimers is circumvented and the homodimers remain activated to stimulate downstream targets such as Src kinase [122, 123].

Another family of resistance mechanisms consists of so-called bypass track pathways. Here, downstream signalling pathways are kept activated by mechanisms "bypassing" the inhibited receptor tyrosine kinase [124]. Examples are the amplification of *MET* or the enhanced stimulation of MET by its ligand, hepatocyte growth factor (HGF) [125]. Similarly, upregulation of EGFR and/or HER3 renders cancer cells resistant to HER2-targeting treatment. In this scenario, more active EGFR/HER3 receptors are available that activate downstream signalling pathways [126]. Moreover, aberrantly activated intracellular kinases of the HER2 signalling pathway can bypass HER2 inhibition. Prominent examples are mutations in the phosphatidylinositol 3-kinase (PI3K) pathway. Such alterations are observed in about 30% of $HER2^+$ breast cancer patients [127]. Patients bearing PI3K catalytic subunit alpha (*PI3KCA*) gene mutations do not benefit from HER2-targeting therapies. As was shown in the randomised phase III EMILIA and Neo-ALLTO trials, where this patient group did not benefit from lapatinib or trastuzumab, respectively [128, 129].

Resistance can also emerge from aberration of the apoptosis pathways. Inhibition of driver oncogenes such as HER2 ultimately leads to apoptosis. Accordingly, expression levels of the pro-apoptotic BH3-only protein Bcl-2-like protein 11 (BIM) are predictive for the response of HER2-overexpressing breast cancer cells to HER2-targeting treatment. Thus, in a small patient cohort where lapatinib was used as a single agent treatment for metastatic $HER2^+$ breast cancer, low BIM expression levels before treatment correlated with inefficient treatment due to inadequate apoptotic response [130, 131]. Moreover, resistance against HER2-targeted treatment in $HER2^+$ breast cancers involves anti-apoptotic Bcl-2 as well as pro-apoptotic BH3-only family members. Accordingly, Bcl-2 is up- and Bax downregulated in trastuzumab-resistant breast cancer cells [132]. As already mentioned, several links between apoptosis and autophagy exist, indicating crosstalk between the two pathways [133]. The aberrant expression of proteins is thus involved in apoptosis influence as well autophagy activity. The anti-apoptotic protein Bcl-2 is often upregulated in tumours. Through binding and subsequent sequestration of the autophagy protein Beclin-1, Bcl-2 can negatively regulate autophagy initiation [27]. High Bcl-2 levels can allow co-existence of low apoptosis as well as low autophagy. The pro-apoptotic Bcl-2 family member Bax can negatively regulate autophagy initiation through caspase-mediated cleavage of Beclin-1 as well [134]. This connection would indicate that activation of apoptosis results in autophagy suppression. Additionally, Bcl-2 family members regulate a non-canonical form of autophagy leading to necroptosis, an apoptosis-independent form of programmed cell death [135]. Here the autophagy machinery serves as a scaffold for the formation of the cell death-inducing signalling complex, the necrosome [136].

Resistance mechanisms against HER2-targeting treatment are multifaceted. Some of them include an aberration of the receptor itself, whereas others bypass HER2 signalling. Examples are the overexpression of other tyrosine kinase receptors or constitutive activation of downstream targets [111]. Resistance may arise from aberrations in the apoptosis pathway as well. As apoptosis and autophagy are interconnected pathways, expression levels of apoptosis proteins may also influence autophagic flux. However, the connection between the pathways is complex and not yet fully understood [69].

HER2 and autophagy – possible candidates for combination therapy?

HER2-targeted therapies are best studied in breast cancer. However, they are also approved for the treatment of $HER2^+$ gastric cancer and tumours of the gastro-oesophageal junction and can be applied off-label for HER2-positive oesophageal adenocarcinoma [137]. Decreased autophagy supports the development of $HER2^+$ breast cancer. Firstly, an association between the loss of the tumour suppressor and autophagy protein Beclin-1 with HER2 gene amplification was found [138]. Secondly, decreased Beclin-1 mRNA expression in mammary tumours is not only associated with worse disease-free survival but is also more common in $HER2^+$ breast cancer [139]. Supporting these notions, a study investigating human and mouse breast cancer cells found that low Beclin-1 mRNA levels correlate with HER2 overexpression, and that *HER2*-amplified tumours exhibit a low autophagy gene expression signature, independent of Beclin-1 mRNA expression. Results from xenograft experiments in this study suggest that in $HER2^+$ tumours autophagy is downregulated even in a Beclin-1 wild type background [140]. Moreover, it was found that HER2 directly interacts with Beclin-1 in breast cancer cells, and that this interaction inhibits autophagy. Disruption of this interaction with Tat-Beclin-1, an autophagy-inducing peptide, caused a cessation of tumour growth in xenograft models [141].

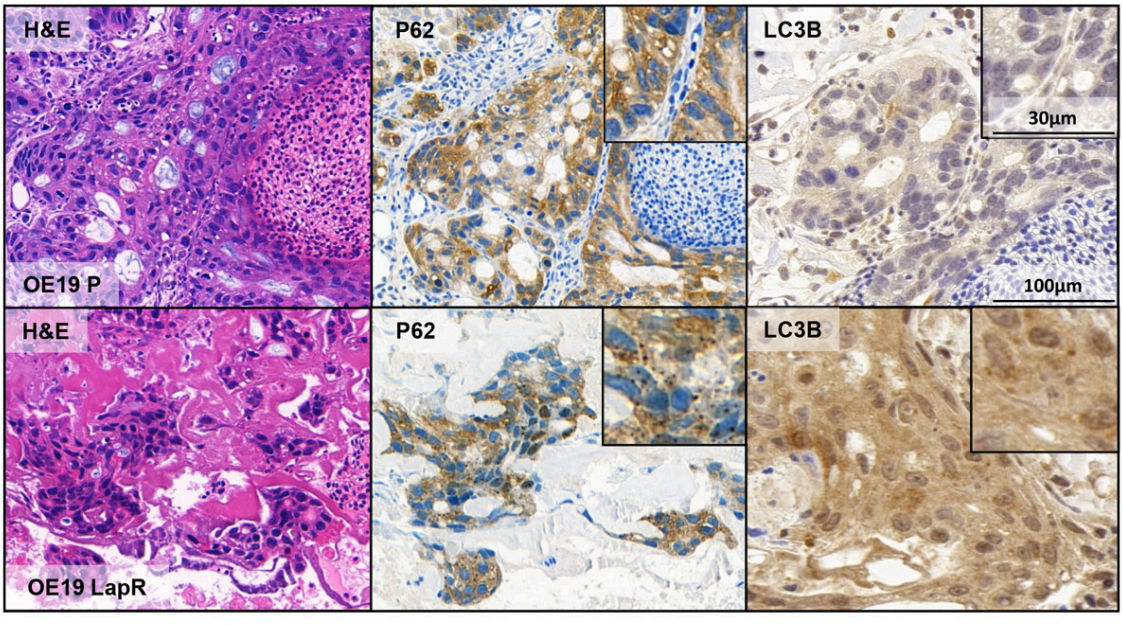
During breast cancer therapy, autophagy has been shown to support resistance to chemotherapeutic agents [142–144]. Similarly, in the context of HER2-targeting treatment, autophagy is mainly discussed as a resistance mechanism. An analysis of a large collection of breast cancer cell lines showed that the transcript levels of ATG12 were upregulated in trastuzumab-non-responsive HER2-overexpressing cells as compared with treatment-sensitive cell lines [145]. Furthermore, trastuzumab-resistant breast cancer spheroids are characterised by increased autophagic activity and show increased sensitivity to autophagy inhibition [146, 147]. Similarly, breast cancer cells rendered resistant to lapatinib exhibited an activation of autophagy and could be re-sensitised to the drug by autophagy inhibition [148, 149]. In our group, we observed a similar phenomenon for *HER2*-amplified oesophageal adenocarcinoma (EAC) cells. In a lapatinib-resistant EAC cell line (OE19LapR) we observed a general upregulation of basal autophagy levels compared to the parental cell line (OE19P). Upon autophagy inhibition, OE19LapR could be re-sensitised to lapatinib treatment to the level of parental cells [150]. We were able to corroborate these findings by growing both cell lines in a chick chorioallantoic membrane (CAM) xenograft assay. The CAM assay is a 3D *in ovo* cell culture model, where tumour cells are grown in a scaffold on an extraembryonic membrane of the chick embryo [151].

Generally, OE19LapR cells formed microtumours that contained more cells, were more vascularised and less necrotic. LC3B and p62 dot formation compared to a homogeneous staining is considered to be an indication for active autophagy in IHC [152]. In the microtumours formed by our two EAC cell lines, OE19LapR and OE19P, higher autophagy levels were indicated by dot-like staining in the OE19LapR tumours compared to the OE19P tumours, where the staining was more homogenous (fig. 3). Conversely, a recent study in gastric cancer cells reported inhibition of autophagic flux upon trastuzumab treatment. A pool of resistant cancer cells showed lower basal levels of autophagy than the parental cells, and autophagy inhibition induced more cell death in the parental cell line than in the trastuzumab refractory cells [153]. Moreover, the activation of autophagy in the context of HER2 inhibitor resistance is discussed as part of a metabolic shift in resistant cells. Trastuzumab-resistant breast cancer cells with upregulated autophagy were shown to have a significantly higher expression of the catalytic subunit of AMPK, AMPKα1 [154, 155]. Cells treated with receptor tyrosine kinase inhibitors activate AMPK as a response to growth factor deprivation, which leads, among other effects, to the inhibition of protein synthesis. This is lethal to *HER2*-amplified breast cancer cells depending on glycolysis [156]. AMPK not only coordinates the adaptive response to ATP depletion but also modulates the activity of autophagy regulators such as ULK1 and mTOR [157]. Thus, increased activity of AMPK could result in a shift to catabolism and prepare cells resistant to HER2 inhibition to activate protective autophagy and overcome the acute bioenergetic crisis resulting from HER2 inhibition [158]. Current data on the role of autophagy in $HER2^+$ tumour formation and possible resistance against HER2 inhibition are not fully conclusive and the role of autophagy in the formation and progression of $HER2^+$ carcinogenesis warrants further studies [159, 160].

On the basis of several preclinical studies demonstrating the antineoplastic effect of autophagy inhibition in combination with a variety of anticancer treatments, first phase I clinical trials studying the safety and antineoplastic effect of the autophagy inhibitors chloroquine and hydroxychloroquine were conducted. In 2014, it was shown that hydroxychloroquine could be safely combined with cytotoxic chemotherapeutics [68, 161, 162]. First clinical results of studies including chloroquine or hydroxychloroquine for anticancer treatment in combination with standard treatment are promising [163]. At the moment there are no clinical trials involving hydroxychloroquine in $HER2^+$ cancers. However, there are several clinical trials investigating the effect of hydroxychloroquine on breast cancer. One of them is the CLEVER pilot trial, which is a phase II trial of hydroxychloroquine in combination with everolimus, an mTOR inhibitor, for the prevention of recurrent breast cancer (NCT03032406). The GLACIER trial is testing the efficacy of gedatolisib (a PI3K/mTOR inhibitor) and hydroxychloroquine on early recurrent breast cancer (NCT03400254). Another study is investigating the efficacy of hydroxychloroquine in metastatico estrogen-receptor-positive breast cancer that has progressed after hormonal therapy (NCT02414776).

Various pieces of evidence suggest a connection between autophagy and HER2. The autophagy-initiating protein Beclin-1 was shown to be low-expressed in $HER2^+$ breast cancer. Moreover, independent of Beclin-1 expression, $HER2^+$ breast cancer cells were shown to exhibit low expression of ATGs. Additionally, the direct interaction of Beclin-1 and HER2 was shown to influence autophagy activity [141]. In the context of resistance to HER2-target-

Figure 3: Increased autophagy in lapatinib-resistant OE19 oesophageal cancer cells. Microtumours developed from OE19 parental (OE19P) and OE19 lapatinib-resistant (OE19LapR) cells are shown in the upper and lower panels, respectively. Depicted from left to right are: H&E, p62 and LC3B staining. Lapatinib-resistant OE19 cells display p62 and LC3B dot formation indicative of increased autophagic activity compared to the parental cell line. The staining intensity of p62 corresponds to a score of 3+ and of LC3B to a score of 2+, according to Schläfli et al. [152].H&E = haematoxylin and eosin; p62 = sequestosome 1, SQSTM1; LC3B = microtubule-associated protein light chain 3B



ed treatment, autophagy is often reported as a resistance mechanism upregulated in resistant cells. Some of these studies showed that autophagy inhibition led to a re-sensitisation to HER2-targeting drugs [148]. The results from previous clinical studies with hydroxychloroquine, indicate that the inhibition of autophagy might be a valuable new avenue for breast cancer treatment. Clearly, there is a need for more specific and potent autophagy inhibitors. Clinical trials investigating the inhibition of autophagy in $HER2^+$ breast cancer are still lacking, but results from ongoing trials in other breast cancer subtypes and the above-mentioned preclinical data might drive research in the direction of combining autophagy inhibition with HER2-targeting treatment in *HER2*-amplified cancers.

Concluding remarks

Generally, the role of macroautophagy in tumour development, progression and resistance formation against cancer therapy is ambivalent [46]. The HER2 oncogene is overexpressed in different tumour types, and drugs specifically targeting this receptor tyrosine kinase have been successfully applied in cancer therapy [98]. However, progression and resistance formation against these drugs ultimately occurs. Resistance mechanisms are multifaceted, and ways to prevent or overcome resistance are urgently needed [159]. Beclin-1, an autophagy initiating protein was reported to directly interact with HER2. Moreover, this interaction was shown to influence autophagy activity [141]. Additionally, Beclin-1 expression was reported to be lower in $HER2^+$ tumours. In the context of resistance to HER2-targeting treatments, autophagy was reported to be upregulated. Even though contradictory data exists, most *in vitro* studies report an upregulation of autophagy in resistant cells, which can be abrogated to some extent by inhibiting autophagy [145, 147, 148]. Still, the understanding of autophagy's role in the development and progression of $HER2^+$ cancers is in its infancy. Future studies deciphering the networks connecting HER2 and autophagy are therefore needed. Two autophagy-inhibiting agents, chloroquine and hydroxychloroquine, are available for clinical application. They have been evaluated for cancer treatment alone and in different combinations in a number of clinical trials. However, it is not yet known whether the beneficial effects observed during hydroxychloroquine and chloroquine treatment are based on their autophagy inhibition function or on their effects on other pathways [64]. Moreover, the inhibitory effect of hydroxychloroquine on the lysosome could lead to defective lysosomal function and thus cause lysosomal storage disease [164, 165]. The development of specific autophagy inhibitors is still in an early phase, but using conditional ATG knockout animals it has been shown that systemic inhibition of essential autophagy genes is possible during a certain therapeutic window [166]. In addition, a better understanding of the crosstalk between autophagy and apoptosis could help to shape the development of new, more specific autophagy targeting agents for antineoplastic treatment. With improved autophagy inhibitors and a better understanding of the processes involved, new quests will undoubtedly arise, such as the search for better and more reliable biomarkers as indicators of macroautophagic flux in human tissue. The combination of LC3B and p62 has been proposed as an approximate approach to characterising different autophagy status in tissue samples [152, 167]. However, other marker proteins such as Beclin-1, ULK1 or ATG5 have also been used to detect autophagy in tissue via IHC (excellently reviewed in [168]). One issue of these attempts to assess autophagy via IHC is that the expression of some autophagy-related proteins does not change upon autophagy induction. Moreover, the expression levels of these proteins are cell-type and tissue-specific, and since most autophagy related genes are also involved in other cellular pathways, an autophagy specific marker or marker combination has not yet been identified. For future studies on autophagy-targeting therapies, however, such biomarkers would be of great value in deciding which tumours should be treated with autophagy-inhibiting or possibly even autophagy-inducing agents.

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Potential competing interests

The authors declare no conflict of interest.

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