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# Targeting PCSK9, through an innovative cVLP-based vaccine, enhanced the therapeutic activity of a cVLP-HER2 vaccine in a preclinical model of HER2-positive mammary carcinoma

Laura Scalambra[1†], Francesca Ruzzi[1*†], Olga Maria Pittino[1], Maria Sofia Semprini[1], Chiara Cappello[1], Stefania Angelicola[1,2], Arianna Palladini[3,4], Patrizia Nanni[1], Louise Goksøyr[5], Cyrielle Fougeroux[5], Manuel L. Penichet[6], Adam Frederik Sander[5,7†] and Pier-Luigi Lollini[1,2*†]

## Abstract

**Background** HER2-targeted therapies have revolutionized the treatment of HER2-positive breast cancer patients, leading to significant improvements in tumor response rates and survival. However, resistance and incomplete response remain considerable challenges. Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibition is a novel therapeutic strategy for the management of dyslipidemia by enhancing the clearance of low-density lipoprotein cholesterol receptors, however recent evidence also shows links between PCSK9 and cancer cells. We present an innovative immunization approach combining capsid virus-like particle (cVLP)-based vaccines against HER2 and PCSK9.

**Methods** The therapeutic activity of the combined vaccine was evaluated in female mice challenged with HER2-positive mammary carcinoma cells. Controls included untreated mice and mice treated with cVLP-PCSK9 and cVLP-HER2 as standalone therapies. Antibodies elicited by vaccinations were detected through ELISA immunoassay. The functional activity of the antibodies was tested in 3D-soft agar assay on human HER2 + + + trastuzumab sensitive and resistant cells.

**Results** Mice vaccinated with cVLP-HER2 + cVLP-PCSK9 displayed tumor regression from the 40th day after cell challenge in 100% of mice remaining tumor-free even 4 months later. In contrast, 83% of mice treated with cVLP-HER2 vaccine alone experienced an initial tumor regression, followed by tumor relapse in 60% of subjects. Untreated mice and mice treated with the cVLP-PCSK9 vaccine alone developed progressive tumors within 1–2 months after cell injection. The combined vaccine approach elicited strong anti-human HER2 antibody responses (reaching 1–2 mg/ml range) comprising multiple immunoglobulins isotypes. cVLP-PCSK9 vaccine elicited anti-PCSK9 antibody responses, resulting in a marked reduction in PCSK9 serum levels. Although the anti-PCSK9 response was reduced when co-administered with cVLP-HER2, it remained significant. Moreover, both cVLP-HER2 + cVLP-PCSK9 and cVLP-HER2 alone

[†]Laura Scalambra and Francesca Ruzzi have contributed equally.

[†]Pier-Luigi Lollini and Adam Frederik Sander jointly supervised this work.

*Correspondence:
Francesca Ruzzi
francesca.ruzzi2@unibo.it
Pier-Luigi Lollini
pierluigi.lollini@unibo.it
Full list of author information is available at the end of the article

induced anti-HER2 antibodies able to inhibit the 3D growth of human HER2 + + + BT-474 and trastuzumab-resistant BT-474 C5 cells. Strikingly, antibodies elicited by the combined vaccination were more effective than those elicited by the cVLP-HER2 vaccine alone in the inhibition of trastuzumab-resistant C5 cells.

**Conclusions** The results indicate that cVLP-PCSK9 vaccination shows adjuvant activity when combined with cVLP-HER2 vaccine, enhancing its therapeutic efficacy against HER2-positive breast cancer and holding promise in overcoming the challenges posed by resistance and incomplete responses to HER2-targeted therapy.

**Keywords** Breast cancer, HER2, Therapy resistance, Cancer progression, PCSK9, Therapeutic cancer vaccines, Virus-like particle (VLP)-based vaccine

## Background

Breast cancer is the most common and deadly cancer diagnosed in women. As a highly heterogeneous neoplasm, it is characterized by multiple tumoral entities that differ in biological and histological properties due to transcriptomic, epigenetic and genetic changes that are associated with varying clinical findings and treatment responses [1–3].

The oncogenic role of the human epidermal growth factor receptor 2 (HER2), exerted by gene mutation, amplification and/or protein overexpression observed in 15–20% of breast cancer and gastric cancer, 10% of bladder cancer and 3% of colorectal cancer, is commonly associated with aggressive behavior and poor prognosis if not treated promptly [4–7].

The tumorigenic features of HER2 are related to its oversignaling, and such dysregulation confers enhanced growth abilities, migration, metastasization and avoidance of apoptosis in favor of proliferation [8]. In the specific case of breast cancer, alterations in the expression or functionality of HER2 result in the diagnosis of a specific subtype usually characterized by higher histological grade, higher invasiveness and unfavorable prognosis, while giving simultaneously the advantage of a specific oncogene to target, overexpressed only in cancer cells [9].

The advent of monoclonal antibodies (mAbs) targeting HER2, such as trastuzumab and pertuzumab, small tyrosine kinase inhibitors (TKIs) and more recently of antibody–drug conjugates (ADCs), significantly improved patient response rate and survival. Unfortunately, a variable proportion of patients eventually present intrinsic or acquired resistance to these treatments which leads to the disease progression [10–14].

Resistance to anti-HER2 therapies in breast cancer involves multiple complex mechanisms that challenge the efficacy of treatments [15–19]. One major contributor is genetic alterations, such as HER2 mutations, which can drive tumor growth even without HER2 overexpression [17]. The activation of alternative signaling pathways that counteract the effects of HER2-targeted therapies [20, 21], or the interaction between HER2 and insulin-like growth factor-I receptor (IGF-1R), that induces phosphorylation and activates the PI3K pathway, play a significant role in therapy resistance [22, 23]. Additionally, specific isoforms of HER2, such as HER2Δ16, exhibit constitutive activation of downstream signaling, and truncated variants like p95HER2, which lack the extracellular domains targeted by HER2 antibodies, can further compromise therapeutic efficacy. However, the exact role of these isoforms in resistance remains a topic of debate [24–26]. Epitope masking might further reduce treatment efficacy by limiting antibody binding [20, 24]. Indeed, lipid rafts were reported to be involved in regulating HER2 signaling and anti-HER2 therapy response. Among other raft-associated proteins, caveolin-1 seems to modulate receptor localization, impairing the sensitivity of cancer cells to targeted therapies [27, 28]. Tumor heterogeneity also poses significant challenges. Variability in HER2 expression within a single tumor can impair the effectiveness of therapies, making targeting all tumor cells more difficult [29–32]. Finally, immune evasion mechanisms undermine the efficacy of anti-HER2 therapies, which depend on immune-mediated responses. An immunosuppressive tumor microenvironment (TME) can impair these responses, reducing treatment effectiveness [17, 18]. Additionally, genetic polymorphisms in the FcγRIII receptor alter its binding affinity to IgG1, thereby diminishing the antibody-dependent cellular cytotoxicity (ADCC) mediated by trastuzumab [33, 34].

The incomplete tumor eradication by HER2-targeted therapy, and consequently the onset of recurrences or failure of therapeutic regimens, can be potentially overcome by the combination of different monoclonal antibodies or by the combination with other immunomodulatory agents, such as immune checkpoint inhibitors and immune-stimulating antibody conjugates [13, 17, 35–41].

From an immunological point of view, an active form of immunization, characterized by a polyclonal and prolonged immunological response could be beneficial to employ a wider range of anti-tumor activities exerted by the different immunoglobulins [42–44]. Anti-cancer responses with such characteristics can be obtained with cancer vaccines [45–49].

A further strategy to enhance targeted therapy efficiency is to interfere with the high energetic and metabolic needs of cancer cells [50–54]. HER2-positive breast cancer resistance to targeted therapies, among others, was found to be associated with an increase in fatty acid (FA) metabolism [55–58]. Several preclinical studies have shown that targeting FA pathways can increase anti-HER2 therapy efficacy or restore treatment response both in breast and gastric cancer [57, 59–62].

The reprogramming of energy metabolism has been identified as a hallmark of cancer and reported as a mechanism of therapy resistance [50, 55]. Cholesterol homeostasis can gain abnormal features during carcinogenesis and cancer spread and has been associated with increased cancer risk [63–70]. From an immunological perspective, high levels of cholesterol inside cells can lead to T-cell exhaustion or prolongation of the resting state [71–74]. In a tumor microenvironment enriched in cholesterol, myeloid suppressor cells have been reported to exert immune-suppressive activities [75–77]. In addition, cholesterol is involved for the formation of immunological synapses, as it may reduce antigen presentation by dendritic cells through the down-regulation of the MHC-I expression and by decreasing their motility [78–80].

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is tightly involved in cholesterol homeostasis, in particular to cholesterol absorption through low-density lipoprotein receptor (LDLR) [81]. This soluble zymogen, primarily secreted by hepatocytes, causes LDLR lysosomal degradation, generating an imbalance in the finely regulated metabolic process that is cholesterol homeostasis. Free LDL cholesterol in the bloodstream is an energetic and architectural source that can be taken-up and metabolized by peripheral cancer cells, while contributing to immuno-suppressive responses in the tumor microenvironment [82].

Indeed, recent evidence demonstrates a link between PCSK9 biological activities and various cancers, including hepatocellular carcinoma (HCC), breast cancer, colorectal cancer (CRC), and prostate cancer (PCa). These changes are frequently associated with increased tumor invasiveness and poorer clinical outcomes [64, 82–84]. Some of the proposed mechanisms involve PCSK9 in cell proliferation, apoptosis and stemness [84, 85]. Furthermore, PCSK9's well-known function in cholesterol metabolism, particularly its regulation of LDLR expression, may indirectly affect cancer growth and metastasis. Cholesterol plays a crucial role in cellular processes linked to tumor development, and PCSK9-mediated alterations in cholesterol homeostasis could contribute to these effects [86, 87]. Another intriguing aspect is the potential link between PCSK9 and immune responses. Expressed by immune cells such as macrophages and lymphocytes, PCSK9 appears to regulate immune cell function. This activity suggests that PCSK9 may influence the tumor microenvironment (TME) by modulating immune surveillance and the body's ability to suppress cancer cell growth [87].

Gain of function (GOF) variants of PCSK9 coding gene or high expression levels of PCSK9 were found to be associated with reduced survival of breast cancer patients, particularly HER2-positive and triple-negative ones (https://kmplot.com/analysis/, accessed on 22nd of April, 2024)[83]. Moreover, a common missense germline variant in PCSK9 (V474I) was recently found associated with breast cancer metastasis and reduced survival in multiple breast cancer patient cohorts [88].

Passive immunotherapy against PCSK9, through monoclonal antibodies, is already approved for the treatment of primary and familiar hyperlipidemia and for the treatment of hypercholesterolemia in statin-resistant patients [89–93]. Active and passive immunological approaches against PCSK9, such as vaccines, are now under preclinical evaluation also for cancer treatment [94–98].

As a cancer vaccine delivery system, virus-like particle (VLP)-based vaccines demonstrated to have great immunogenic potential due to their high-density epitope display and their ability to emulate native bacteriophages and viruses in their ability to trigger humoral and cellular immune responses [99–102], even in preclinical settings of tumor prevention and therapy of several cancer types [102–104], including HER2-positive breast cancer [42, 105–108].

Thus, we sought to investigate whether the combination of two cVLP-based vaccines against HER2 and PCSK9, which separately demonstrated to be effective in triggering neutralizing immune responses [42, 94, 105], could enhance tumor eradication and prolong survival in a preclinical model of HER2-positive breast cancer.

## Methods

### Vaccine formulation

The system employs a Tag/Catcher conjugation system: AP205 phage capsid cVLP is conjugated with Catcher-HER2$^{ECD}$ (human) or Tag-PCSK9 (mouse). Design, expression and purification of cVLP-HER2 and cVLP-PCSK9 vaccines were performed as previously reported [42, 94].

### Cell lines

MamBo89HER2$^{stable}$ cell line was established from a spontaneous mammary carcinoma of a transgenic human HER2 female mouse (FVBhHER2) bred in our animal facility (breeders were originally received from Genentech, South San Francisco, CA, USA) [30].

Mambo89HER2$^{stable}$, which expresses surface human HER2 at levels comparable to those of 3 + human breast cancer cell lines like BT-474, represents a good preclinical model to test immunological therapies directed against HER2 in immunocompetent mice [30, 42]. MamBo89HER2$^{stable}$ cell line was cultured in DMEM (Thermo Fisher Scientific, Monza, Italy) supplemented with 20% of fetal bovine serum (FBS), 100 U/mL penicillin and 10 µg/mL streptomycin (Thermo Fisher Scientific, Monza, Italy), 30 µg/ml bovine pituitary extract (Corning Life Sciences, USA) and 0.5% v/v MITO Serum Extender (Corning Life Sciences, USA) [30].

Human HER2-positive breast cancer cell line BT-474 (HER2 + + +) and its trastuzumab-resistant clone C5 (from ATCC) were routinely cultured in RPMI (Thermo Fisher Scientific, Monza, Italy) supplemented with 10% FBS, 100 U/mL penicillin and 10 µg/mL streptomycin. BT-474 cell line (from ATCC) was kindly provided by Dr. S. Pupa (Istituto Nazionale dei Tumori, Milan, Italy);

All cell lines were cultured at 37 °C in a humidified 5% $CO_2$ atmosphere and were split once or twice a week according to density, using 0.05% trypsin–EDTA (Thermo Fisher Scientific, Monza, Italy).

#### Mice, tumor cells challenge, vaccination and bleedings

FVB female mice were purchased from Charles River (Calco, Lecco, Italy).

18-week-old virgin FVB female mice were challenged with $5 \times 10^6$ MamBo89HER2$^{stable}$ cells in the mammary fat pad (m.f.p) of the fourth mammary area.

Vaccines were formulated with AddaVax (Invitrogen, Thermo Fisher Scientific, Waltham, USA), as adjuvant 1:1. The vehicle group (also referred as 'untreated') received PBS alone.

The standard dose of vaccine was 10 µg and 5 µg per mouse per administration for cVLP-HER2 vaccine and cVLP-PCSK9 respectively, as reported in previous works [42, 94]. Vaccines were administered intramuscularly (i.m.) in the left hind leg. Vaccinations started two weeks after cell challenge and mice received 6 bi-weekly vaccine administrations.

Mice were bled before each vaccination and two weeks after the last vaccination. Sera were stored at -80 °C and tested through immunological assays.

Tumors were measured with calipers twice a week; tumor volume was calculated as $\pi/6*(\sqrt{a}b)^3$ where $a$ = maximal tumor diameter and $b$ = maximal tumor diameter perpendicular to a. Mice were euthanized if they showed any signs of distress or when tumor volume reached 2 $cm^3$.

All mice were monitored daily and weighed twice weekly.

All in vivo experiments were performed according to Italian and European laws and were authorized by the Italian Ministry of Health (letter 714–2017-PR).

#### Enzyme-linked immunosorbent assay (ELISA)

Serum anti-human HER2 IgG levels were quantitatively measured by ELISA immunoassay.

Immunoplate Nunc Maxisorp 96-well microplates (Merck, Darmstadt, Germany) were coated overnight with the HER2$^{ECD}$ or with PCSK9 protein at 1 µg/mL in carbonate bicarbonate buffer (Merck, Darmstadt, Germany) [42, 94].

A standard curve (from 0.04 to 30 ng/mL) with mouse monoclonal antibody 4D5 against human HER2 (Genentech, Roche, San Francisco, USA) was run in parallel.

The following horseradish peroxidase (HRP)-labeled goat anti-mouse Ig antibodies, all from Thermo Fisher Scientific, were used for the detection of: total IgG (1:30,000 dilution), IgM (1:10,000 dilution), IgG1 (1:10,000 dilution), IgG2a (1:5000 dilution), IgG2b (1:10,000 dilution), IgG3 (1:5000 dilution).

PCSK9 protein level in mouse sera was evaluated by Mouse Proprotein Convertase 9/PCSK9 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

Optical density (OD) was determined with a microplate reader (Sunrise, Tecan, Switzerland).

#### 3D-agar colony growth inhibition

In vitro sensitivity of human breast cancer BT-474 (HER2 + + +, trastuzumab-sensitive) and BT-474 C5 (HER2 + + +, trastuzumab-resistant) cells to cVLP-HER2 and cVLP-PCSK9-induced antibodies were evaluated in a 3D-agar colony growth assay.

As described in detail previously [109], cells were seeded at 500 cells/well in 24-well plates (Corning Life Sciences, USA) in RPMI supplemented with 10% FBS + 0.33% agar (Lonza Bioscience Solutions, Siena, Italy) with mouse sera diluted 1:100 or trastuzumab (kindly provided by Genentech) diluted at the same concentration as anti-HER2 antibodies in vaccinated mouse sera.

Colonies (diameter > 90 µm) were counted under an inverted microscope equipped with an ocular micrometer in dark-field, 14–30 days after seeding.

#### Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 9.2.0) (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com).

The Kaplan–Meier tumor-free survival curve, as a method for estimating the survival function from time datapoints, was applied to events of progression (tumor

positivity) after an initial tumor regression. Events occurred when no regression was achieved or when a specific tumor volume ($\geq 0.01$ $cm^3$) was reached after an initial regression. The comparison of the Kaplan–Meier curves was carried out with Log-Rank (Mantel-Cox) test, for detecting differences between groups when the risk of an event is not equally distributed.

Anti-PCSK9 antibodies, serum PCSK9 protein levels and their percentage of inhibition were compared with the unequal variance *t*-test, also called Welch's *t*-test (unpaired). The unpaired Welch's *t*-test compares the means of two unmatched groups (e.g. vehicle and cVLPs, in our case), without the assumption that their standard deviations are similar.

The number of colonies in the 3D agar colony inhibition assay was compared with Student's *t*-test (unpaired).

## Results

### cVLP-HER2 and cVLP-PCSK9 vaccines as therapeutic strategies of HER2-positive mammary carcinoma

The therapeutic activity of the combination of cVLP-HER2 and cVLP-PCSK9 vaccines was evaluated against mammary carcinomas induced by the injection of MamBo89HER2$^{stable}$ cells in the mammary fat pad of syngeneic FVB female mice, and compared to the control groups treated with cVLP-HER2 and cVLP-PCSK9 alone or untreated.

Vaccines, formulated with AddaVax adjuvant in a 1:1 ratio, were administered bi-weekly starting 2 weeks after cell challenge, for a total of 6 immunizations (Fig. 1A).

Untreated mice and those treated with the cVLP-PCSK9 vaccine alone developed progressive tumors within 1–2 months after cell injection, whereas 83% of mice vaccinated with cVLP-HER2 and 100% of cVLP-HER2 + cVLP-PCSK9 showed tumor regression from the 40th day after cell challenge (Fig. 1B-D). While both cVLP-HER2 and cVLP-HER2 + cVLP-PCSK9 approaches significantly increased mice survival if compared to untreated and cVLP-PCSK9 treated group, all mice treated with the vaccine combination remained tumor-free even 4 months later, whereas 60% of cVLP-HER2 vaccinated mice experienced tumor relapse (Fig. 1B). Thus, the addition of cVLP-PCSK9 vaccine significantly increased the long-term therapeutic efficacy of cVLP-HER2 (Fig. 1B).

### Anti-HER2 antibody response

In FVB mice, cVLP-HER2 + cVLP-PCSK9 vaccination elicited strong (1–2 mg/mL) anti-human HER2 antibody responses (Fig. 2A), comprising different mouse IgG isotypes subclasses, comparable to the ones elicited by cVLP-HER2 vaccination alone. In particular, the polyclonal anti-HER2 antibody response included the IgG subclasses with the strongest antitumoral effect (i.e. IgG2a, IgG2b and IgG3) (Fig. 2B) [110, 111]. The prevalent IgG isotype was IgG1, closely followed by IgG2a and IgG2b, in both the combination therapy and cVLP-HER2 treated groups (Fig. 2B).

### Anti-PCSK9 antibody response and PCSK9 protein modulation in mouse sera

The binding activity of anti-PCSK9 antibodies to full-length PCSK9 was investigated through ELISA assay.

While anti-PCSK9 antibody levels could barely be detected in the vehicle and cVLP-HER2 treated groups, cVLP-PCSK9 vaccine alone elicited a 137-fold increase in anti-PCSK9 antibody titers, compared to the control group. The anti-PCSK9 response reached a smaller but still significant increase (sevenfold) compared to vehicle treated mice (Fig. 3A, B).

The biological effect of the vaccine-induced antibodies was investigated by measuring PCSK9 protein levels in mice sera, resulting in an overall negligible inhibition in the vehicle group and cVLP-HER2 group (Fig. 3C, D). According to PCSK9 antibodies levels, PCSK9 protein levels were significantly reduced in the cVLP-PCSK9 treatment group, while the combination of cVLP-PCSK9 with cVLP-HER2 was associated with an overall less marked inhibition and opsonization of PCSK9; in this group there was a more heterogeneous protein inhibition, as two-thirds of mice presented a decrease in PCSK9 protein levels (data not shown).

### Inhibition of human HER2-positive breast cancer cells growth in 3D cultures

To further evaluate the functional activity of the antibodies induced by the vaccines, their inhibitory effect was tested in a 3D-soft agar colony formation assay on human HER2 + + + trastuzumab-sensitive, BT-474, and on its trastuzumab-resistant clone BT-474 C5.

Mouse serum from mice immunized with vehicle, cVLP-HER2, cVLP-PCSK9 and cVLP-HER2 combined with cVLP-PCSK9 vaccines were diluted in soft-agar layers. As technical controls, untreated tumor-free mouse sera and trastuzumab were diluted in a similar concentration as the immune sera in the soft-agar layers.

As expected, the BT-474 cell line was significantly inhibited by trastuzumab, whereas the BT474 C5 clone was not inhibited (Fig. 4).

The anti-human HER2 antibodies elicited by cVLP-HER2 + cVLP-PCSK9 and cVLP-HER2 alone significantly impaired 3D-colony formation of both BT-474 and BT-474 C5 cells. Moreover, antibodies elicited by the combined vaccination were more effective than those elicited by the cVLP-HER2 vaccine alone in the inhibition of BT-474 C5 (Fig. 4).

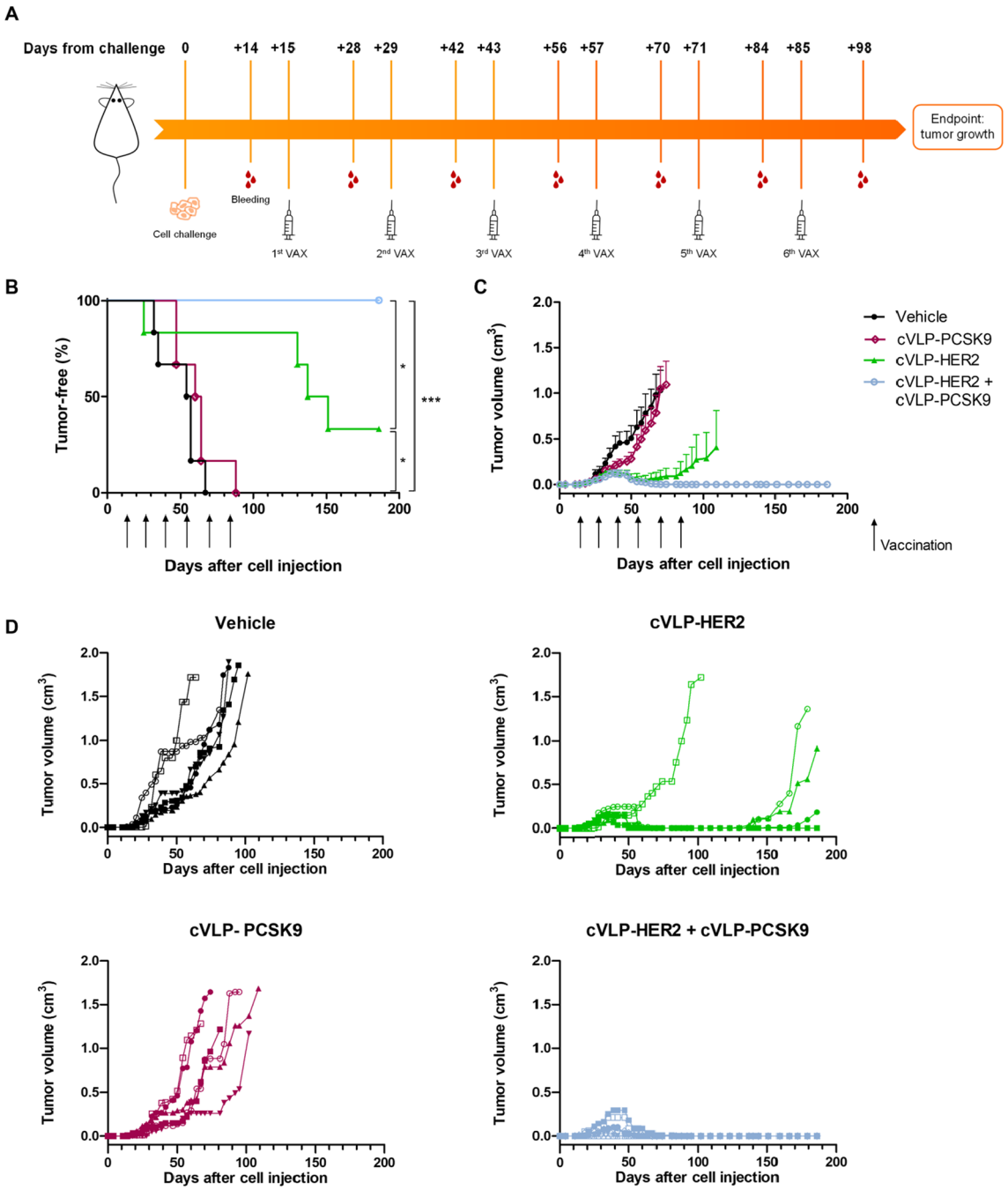


**Fig. 1** Therapeutic efficacy of cVLP-PCSK9 and cVLP-HER2 vaccines as standalone or combined approach. **A** Experimental design; **B** Kaplan–Meier tumor free survival plot, n = 6; *$p < 0.05$; ***$p < 0.01$; long-rank test; **C** Tumor growth curves, each point represents the mean (and SEM) of all mice in each group, n = 6; **D** Single mice growth curves of each treated group

## Discussion

In this work, we combined a cVLP-based vaccine against human HER2, which previously demonstrated strong immunogenicity and anti-tumoral activities in preclinical mouse model of HER2-positive breast cancer [42, 105], with a second cVLP-based vaccine directed against mouse PCSK9, developed to manage cholesterol-related disease [94].

Combining cVLP-PCSK9 and cVLP-HER2 vaccines resulted in more efficient and rapid tumor regression and in a significantly higher tumor-free survival, compared to cVLP-HER2 vaccine alone.

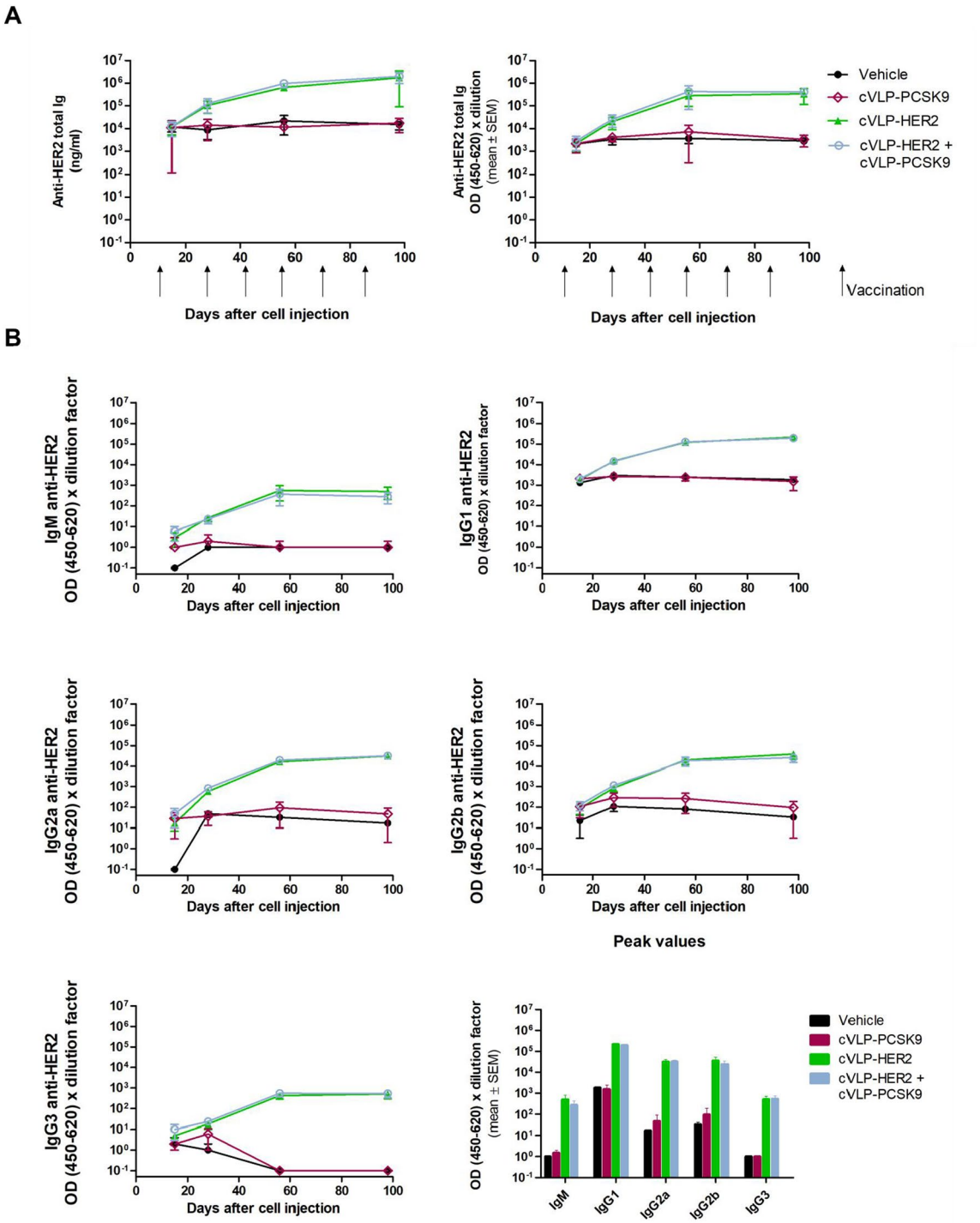


**Fig. 2** Anti-HER2 polyclonal antibody response elicited by cVLP-HER2 vaccine. **A** Anti-HER2 antibody titers measured by ELISA on human $HER2^{ECD}$, n = 6. Each point represents the mean (and SEM) of mouse groups shown in Fig. 1; **B** HER2 specific IgM and IgG isotypes elicited by cVLP-HER2 vaccinations, n = 2–4. Each point represents the mean (and SEM). Histogram reports the mean (and SEM) peak value of each isotype after the last vaccination

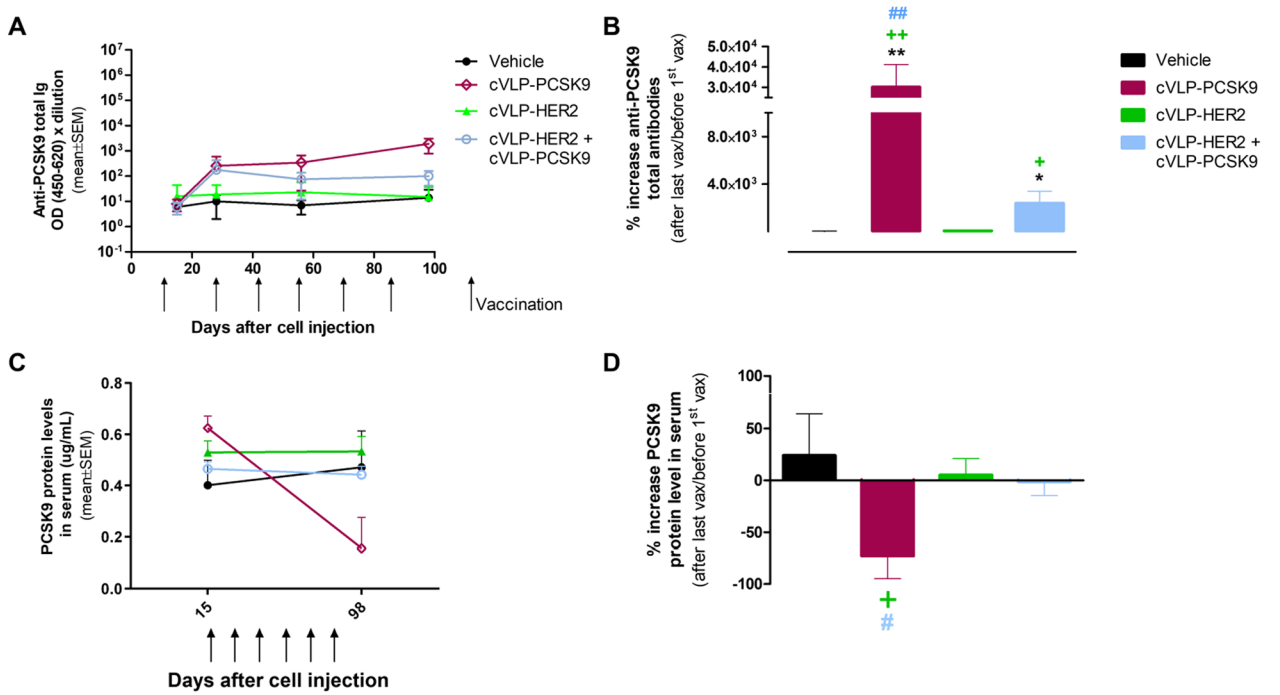


**Fig. 3** Anti-PCSK9 responses induced by cVLP-PCSK9 vaccine. **A** Mean (and SEM) of anti-PCSK9 antibody titers measured by ELISA represented as logarithm of Optical Density (OD) value multiplied by dilution factor of the serum sample, n = 6. Vehicle vs. cVLP-PCSK9, at least $p < 0.01$, from day 56; Vehicle vs. cVLP-HER2 + cVLP-PCSK9, $p < 0.05$, from day 98; cVLP-HER2 vs. cVLP-PCSK9, at least $p < 0.05$, from day 56; cVLP-PCSK9 vs. cVLP-HER2 + cVLP-PCSK9, at least $p < 0.05$, from day 98; cVLP-HER2 vs. cVLP-HER2 + cVLP-PCSK9, $p < 0.05$, day 98. All statistics were carried out by Welch *t*-test. **B** Percentage of the increase of anti-PCSK9 total antibodies after the last vaccination. Histogram reports mean (and SEM) of each group. *$p < 0.05$ vs Vehicle, +$p < 0.05$ vs cVLP-HER2, #$p < 0.05$ at least vs cVLP-HER2 + cVLP-PCSK9 by Welch's *t*-test; **C** PCSK9 protein levels in mouse serum (µg/mL), each point represents the mean (and SEM) for each experimental group, n = 5–6. **D** Percentage of the increase of anti-PCSK9 protein inhibition after the last vaccination. Histogram reports mean (and SEM) of each group. + p < 0.05 vs cVLP-HER2, #p < 0.05 at least vs cVLP-HER2 + cVLP-PCSK9 by Welch's t-test

In our previous studies [105], we achieved a tumor-free survival of about two years by treating mice with the cVLP-HER2 vaccine combined with Montanide ISA51 as adjuvant. In the present study, we aimed to assess the potential adjuvant effect of the cVLP-PCSK9 vaccine by testing the cVLP-HER2 vaccine under suboptimal conditions. Specifically, we challenged mice with a different cell line and injection dose, using MamBo89HER2$^{stable}$ cells instead of D16_BO_QD cells. Based on our previous findings, AddaVax elicited antibody titers that were five times lower than those achieved with Montanide ISA51 when combined with the cVLP-HER2 vaccine (data not shown), thus we administered the cVLP-HER2 vaccine with AddaVax, in order to not reach the cVLP-HER2 maximal therapeutic efficacy and to shed light on the possible adjuvant effect exerted by the cVLP-PCSK9 vaccine.

The combination of cVLP-HER2 and cVLP-PCSK9 induced strong anti-human HER2 antibody response, slightly higher than those induced by cVLP-HER2 alone, while anti-PCSK9 antibodies levels were reduced in the combined immunization approach if compared to the single treatment with cVLP-PCSK9. Overall, cVLP-PCSK9 alone elicited a humoral response that inhibited PCSK9 protein levels and, less prominently, in combination with cVLP-HER2. The lower, but still significant compared to controls, anti-PCSK9 response observed in mice receiving the cVLP-PCSK9 combined with cVLP-HER2 could be linked to the immunodominance of HER2 or to potential vaccine interferences [112]. To further enhance cVLP-PCSK9 antibody response when administered with cVLP-HER2, different immunization schedules will be evaluated.

The main aim of the present study was to investigate the therapeutic activity of cVLP-HER2 + cVLP-PCSK9 against HER2-positive mammary carcinomas, but we also had the opportunity to preliminarily evaluate its safety in mice. Weight curves of unvaccinated and vaccinated mice overlapped (Supplementary Figures S1 and S2). As judged from the constant monitoring of the mice, vaccines either alone or combined did not compromise the general health status of mice. Moreover, no signs of suffering or distress were reached according to the *humane endpoints*. At necropsy, liver and kidney tissues

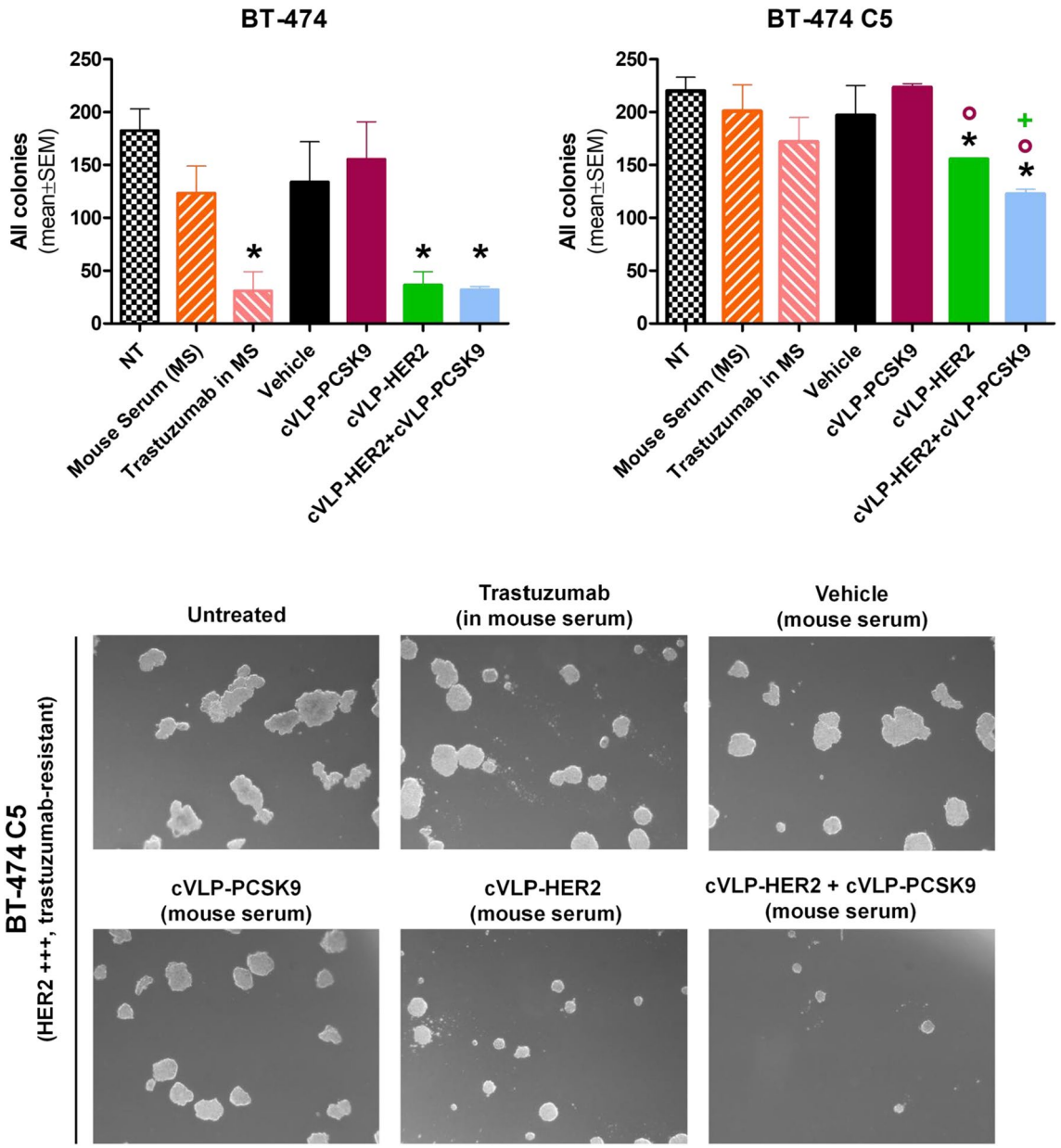


**Fig. 4** Inhibition of human breast cancer cell 3D agar colony growth by antibodies elicited by vaccinations. Upper panel**,** colony inhibition assay on BT-474 (HER2 + + +, trastuzumab sensitive) and BT-474 C5 (HER2 + + +, trastuzumab resistant), n = 2. Each bar represents the mean (and SEM) number of colonies larger than 90 µm as counted in two independent cultures with the aid of a micrometer. *$p < 0.05$ vs untreated, °$p < 0.05$ vs cVLP-PCSK9, +$p < 0.05$ vs cVLP-HER2, by t student's test, unpaired. Lower panel, representative pictures of live agar colonies were acquired with an inverted microscope (dark-field, 25X)

were not altered and macroscopically no signs of local toxicity (e.g. redness, swelling or accumulation of suspension in the hind leg) were reported.

To better evaluate the functional activity of antibodies induced by the vaccines, their inhibitory activity was investigated on human HER2 + + + breast cancer cells BT-474 (trastuzumab-sensitive) and on its trastuzumab-resistant clone BT-474 C5, in three-dimensional colonies in soft-agar. As expected, anti-HER2 antibodies elicited by cVLP-HER2 + cVLP-PCSK9 vaccines exerted great inhibitory abilities on the HER2-positive trastuzumab-sensitive cell line, similar to the ones elicited by cVLP-HER2 alone. Interestingly, on the trastuzumab-resistant cell line, antibodies elicited by the

combination therapy showed a higher inhibitory effect than cVLP-HER2 alone antibodies, suggesting that this therapeutic regimen might be more effective in managing heterogeneous human HER2-positive tumors in which trastuzumab-resistant and trastuzumab-sensitive subclones coexist.

Mechanistically, the higher therapeutic effect of the cVLP-HER2 and cVLP-PCSK9 combined immunization protocol was not strictly related to the inhibition of PCSK9, as more information about the anti-tumor activity of the inhibition of PCSK9 in our preclinical model need to be collected. We hypothesize that the great in vivo inhibition showed by this therapeutical approach is strictly related to the anti-human HER2 antibodies induced both by the combined approach and by cVLP-HER2 alone. In our previous results [113], we saw that the inhibition of HER2/neu carcinogenesis depends on antibody production which can be related to B cells and IFN-γ release. Thus, we may expect that T cell depletion results in a reduced IFN-γ release and consequently in a lower antibody production which can impair vaccine therapeutic efficacy. While not reaching statistical significance, antibodies induced by the combined vaccination approach are higher than those induced by cVLP-HER2 alone, contributing to the stronger anti-tumoral effect together with an increase in antibodies' affinity and avidity. The in vitro experiments on human cell lines confirmed that an immune mechanism by which anti-HER2 antibodies elicited by vaccines exert their anti-tumor activity is through direct inhibition of HER2-positive tumor cells.

Moreover, the induction of a polyclonal antibody response against HER2, involving in particular the IgG2a subtype, could indicate the activation of a T helper type 1 (Th1) immune response, able to enhance tumor antigens presentation, activation of cytotoxic T lymphocytes or to directly kill tumor cells by death-receptor activation by cytokines on cancer cells [114–116].

In addition, in HER2 overexpressing tumors, where the signaling receptor is colocalized with cholesterol-rich signaling niches called lipid rafts, the role of cholesterol and PCSK9 is not only on a metabolic level, but also on a signaling level, as these lipid structures can affect HER2 stability on the cell membrane and its internalization after anti-HER2 targeted treatments [28, 40, 117].

Another potential mechanism behind the increased antitumoral effect exerted by the combination of the two vaccines could be the modulation of low-density lipoprotein receptor (LDLR). Partial inhibition of PCSK9 may have lifted the blockage on LDLR on lymphocytes, leading to enhanced TCR recycling and activation of CD8 + effector T cells [118]. This effect is independent of cholesterol levels, but depends on PCSK9 biological functions [118]. Therefore, it is crucial to gain a broader understanding of the PCSK9 protein level threshold associated with its functionality.

Indeed, the enhanced antitumoral effect of the combination therapy could be associated to an impairment of MHC-I degradation, which was reported to be caused by binding with PCSK9 [119, 120]. Thus, the inhibition of PCSK9 could have enhanced antigen-presenting abilities and activated cell-mediated immune responses. Deeper analyses are necessary to understand whether the effect on tumor growth is attributed to the effects of PCSK9 on the immune system.

In summary, our results suggest that anti-PCSK9 treatments can enhance anti-HER2 therapies efficacy, also against trastuzumab-resistant cells, confirming that VLP-based vaccines are a valid therapeutic strategy able to induce strong humoral responses and antitumor effects.

## Conclusions

HER2-targeted therapies have revolutionized the treatment of HER2-positive breast cancer patients; however, resistance and tumor relapse remain considerable challenges.

Our results showed that cVLP-PCSK9 vaccine had adjuvant activity when combined with cVLP-HER2 vaccine, enhancing its therapeutic efficacy against HER2-positive breast cancer and mice tumor-free survival.

On a trastuzumab-resistant cell line, the antibodies elicited by the combined therapy showed a higher inhibitory effect than ones elicited by cVLP-HER2 alone, holding promise in overcoming the challenges posed by resistance and incomplete responses to HER2-targeted therapy.

### Abbreviations

| | |
|---|---|
| ADC | Antibody–drug conjugate |
| cVLP | Capsid virus-like particle |
| ECD | Extracellular domain |
| FA | Fatty acid |
| FBS | Fetal bovine serum |
| GOF | Gain of function |
| HER2 | Human epidermal growth factor receptor 2 |
| LDLR | Low-density lipoprotein receptor |
| mAb | Monoclonal antibody |
| MHC | Major histocompatibility complex |
| OD | Optical density |
| PCSK9 | Proprotein convertase subtilisin/kexin type 9 |
| TCR | T-cell receptor |
| Th1 | T-helper type 1 |
| TKIs | Tyrosin-kinase inhibitors |

## Supplementary Information

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Supplementary material 1.

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**Availability of data and materials**
Not applicable.

## Declarations

**Ethics approval and consent to participate**
All in vivo experiments were performed according to Italian and European laws and were authorized by the Italian Ministry of Health (letter 714–2017-PR).

**Consent for Publication**
Not applicable.

**Competing interests**
L.G., C.F. and A.F.S. are listed as co-inventors on a patent application covering the Delivery of a cVLP-based modular vaccine platform in a nucleic acid platform (P5856PC00). Employees of AdaptVac (L.G., C.F. and A.F.S.), a company commercializing virus-like particle display technology and vaccine, including several patents.

**Author details**
[1]Department of Medical and Surgical Sciences (DIMEC), University of Bologna, Bologna, Italy. [2]IRCCS Policlinico Di Sant'Orsola University Hospital, Bologna, Italy. [3]Department of Molecular Medicine, University of Pavia, Pavia, Italy. [4]Medical Oncology Division, IRCCS Policlinico San Matteo, Pavia, Italy. [5]AdaptVac Aps, Copenhagen, Denmark. [6]Division of Surgical Oncology, Department of Surgery and Department of Microbiology, Immunology and Molecular GeneticsThe Molecular Biology InstituteJonsson Comprehensive Cancer Centre, University of California, Los Angeles (UCLA), CA, USA. [7]Centre for Translational Medicine and Parasitology, Department for Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark.

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