Results from a phase I clinical study of the novel li-Key/HER-2/neu(776-790) hybrid peptide vaccine in patients with prostate cancer

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ABSTRACT

Purpose
Active immunotherapy is emerging as a potential therapeutic approach for prostate cancer (PC). We conducted the first phase I trial of an i-Key/HER-2/neu(776-790) hybrid peptide vaccine (AE37) with recombinant GM-CSF as adjuvant in patients with HER-2/neu+ PC. The primary end-points of the study were to evaluate toxicity and monitor patients' immune responses to the vaccine.

Experimental design
Thirty-two HER-2/neu+, castrate-sensitive (CS) and castrate-resistant (CR) PC patients were enrolled. Of these, 29 patients completed all six vaccination cycles with AE37. Immunologic responses in the total patients' population were monitored by DTH and IFN-γ ELISPOT and intracellular staining. Regulatory T-cell (Treg) frequency and plasma HER-2/neu and TGF-β levels were also determined. Immunological responses were also analyzed among groups of patients with different clinical characteristics. Local/systemic toxicities were monitored throughout the study.

Results
Toxicities beyond grade 2 were not observed. Seventy-five percent of patients developed augmented immunity to the AE37 vaccine and 65% to the unmodified AE36 peptide as detected in the IFN-γ-based ELISPOT assay. Intracellular IFN-γ analyses revealed that AE37 elicited both CD4+ and CD8+ T-cell responses. Eighty percent of the patients developed a positive DTH reaction to AE36. Additionally, significant decreases could be detected in circulating Treg...
frequencies, plasma HER-2/neu and serum TGF-β levels. Patients with less extensive disease developed better immunological responses upon vaccination.

**Conclusion**

AE37 vaccine is safe and can induce HER-2/neu-specific cellular immune responses in patients with CSPC and CRPC, thus emphasizing the potential of AE37 to target HER-2/neu for the immunotherapy of prostate cancer.
TRANSLATIONAL RELEVANCE

Tolerance mechanisms against (self) tumor proteins hamper the generation of robust antitumor responses and thus represent a major limitation for tumor immunotherapy. One method to overcome such tolerance mechanisms is to increase the immunogenicity of synthetic peptide vaccines containing tumor protein epitopes. In a phase I clinical trial, we provided immune activation signals through an Ii-Key/HER-2/neu(776-790) (AE37) hybrid peptide/GM-CSF vaccine in patients with prostate cancer. We describe the safety and vaccine-induced immunity of this combination in these patients. Our results show that the AE37 hybrid peptide/GM-CSF vaccine strategy is safe and effective in eliciting an immune response against the HER-2/neu protein.

These observations suggest that further investigation of AE37 in a randomized phase II clinical trial, where clinical benefit in terms of evaluating time to disease progression can be assessed, is warranted.
INTRODUCTION

Despite conventional treatments for prostate cancer (PC), such as surgical resection and radiotherapy, many patients will finally develop metastases. Hormone deprivation is initially effective in the majority of patients with metastatic disease (1). Eventually, however, PC patients will relapse due to progression of PC towards hormone-independent growth of the tumor. Unfortunately, advanced PC responds poorly to chemotherapeutic agents of which taxane-based treatments are nowadays considered as the common treatment of CRPC (2). Therefore, novel approaches are required for the prevention and/or effective management of patients with advanced PC.

Prostate glands are frequently diffusely infiltrated with both CD4^+ and CD8^+ T cells (3, 4) suggesting that PC may represent an attractive target for immunotherapy. Indeed, several prostate-specific gene products have been reported that may function as tumor/tissue antigens (5-7). Active immunotherapies in PC have employed a variety of methods to augment immune responses to PC-associated antigens. Of these the most promising include PROSTVAC and APC8015 (Provenge). PROSTVAC-VF comprises two recombinant viral vectors, each encoding transgenes for PSA. In two phase II studies PROSTVAC-VF immunotherapy was well tolerated and associated with a significant reduction in the death rate and an improvement in median OS in men with metastatic CRPC (8, 9). APC8015 is a novel immunotherapeutic, which consists of autologous dendritic cells pulsed ex vivo with PA2024, a recombinant fusion protein consisting of GM-CSF and prostatic acid phosphatase, as an immunogenic agent. Two phase III randomized clinical
trials have demonstrated a survival benefit in patients with metastatic hormone-refractory prostate cancer (10, 11). A novel method to augment potency of MHC class II epitope peptide presentation has now become available in the form of li-Key/MHC class II hybrid peptides (12). Such li-Key hybrid peptides contain an immunoregulatory segment of the li protein that catalyzes direct charging of MHC class II epitopes to the peptide-binding groove, circumventing the need for intracellular epitope processing (13). The shortest active sequence of the li immunoregulatory region consists of 4 amino acids [LRMK (li-Key peptide)] (12). Covalent linkage of this li-Key segment to MHC class II epitopes significantly augments antigen presentation (13-16). Work from our laboratory has shown that the HER-2/neu(776-790) epitope serves as a compelling tumor antigen and that CD4+ T cells primed with the synthetic HER-2/neu(776-790) peptide help autologous CTL for increased antitumor activity (17-19). In a series of studies (14, 17-20) we have shown that the li-Key/HER-2/neu(776-790) hybrid (AE37) induces more potent immunologic responses both in vitro and in vivo compared to the non-modified HER-2/neu(776-790) peptide (AE36).

Recently, Holmes et al (21) conducted a phase I study of the AE37 hybrid peptide in HER-2/neu-positive breast cancer patients and demonstrated strong immunologic responses to the vaccine.

In prostate cancer, several studies have reported increased HER-2/neu expression in patients with clinically localized, as well as more advanced hormone refractory disease (22-24). In addition to stimulating cell division, HER-2/neu also confers an increased malignant potential to prostate cancer cells by activating the androgen receptor pathway in the absence of androgen (25), thus contributing to the development of CRPC. Indeed, clinical studies have shown
that progression of prostate cancer toward androgen independence is characterized by a gradual increase in HER-2/neu expression (26, 27). Therefore, HER-2/neu targeting represents a promising therapeutic intervention for prostate cancer patients. Active immunotherapy provides the added benefit in that specifically activated immune cells can recognize and kill tumor cells expressing lower levels of HER2/neu than are required for recognition by Herceptin. We report here the results of the first trial with AE37-immunized prostate cancer patients.
MATERIALS AND METHODS

Patient population, clinical protocol and study design.

The phase I clinical trial protocol was approved by the Hellenic National Organization for Medicines (EOF) under the investigational number IS107/2006, EudraCT 2006-003299-37. Patients’ primary tumors were evaluated by immunohistochemistry (IHC) for HER-2/neu expression (27). After proper counselling and written informed consent, CSPC and CRPC patients with HER-2/neu+ (IHC score 1+ to 3+), non metastatic and metastatic disease and ECOG 0 or 1, were eligible. Before vaccination, patients were skin tested with C. Albicans (Lofarma, Italy) and considered immunocompetent if they reacted to the antigen. Exclusion criteria included patients with ECOG ≥2, active infection, severe cardiovascular comorbidity, acute/chronic HBV, HCV, HIV, HIV seropositivity, diagnosis of other primary solid/hematologic malignancy and any pathological comorbidity affecting patients’ compliance in the proposed clinical protocol (e.g., mental disorder) and immunologically inactive (negative skin test). Patients were assigned to receive 500 μg of the AE37 vaccine mixed with 125 μg of GM-CSF at each vaccination cycle. The doses for both AE37 and GM-CSF used were found to be optimal in a phase I study with disease-free, node-negative breast cancer patients (21) where a similar vaccination schedule was followed.

Vaccine

The Ii-Key/HER2/neu(776-790) hybrid peptide, AE37 (Ac-LRMKGVSPYVSRLGICL-NH2) was produced in accordance with current
federal guidelines for good manufacturing practices by NeoMPS Inc (San Diego, CA). Peptide purity (>95%) was verified by high performance liquid chromatography and mass spectrometry. Sterility testing was carried out by the manufacturer. The vaccine was prepared as previously described (21). Briefly, 500μg of the lyophilized peptide were reconstituted in 0.5mL of sterile saline and mixed with 125μg granulocyte-macrophage colony-stimulating factor (GM-CSF) (Berlex, Seattle, WA) in 0.5mL. The 1.0mL inoculation was split and administered intradermally at two sites 5cm apart in the same extremity. All patients received 6 monthly vaccinations with the AE37 vaccine. GM-CSF was reduced by half serially in subsequent inoculations when local reactions of ≥100 mm in diameter (21) were observed. If the latter patients continued with robust local reactions without GM-CSF, then the amount of the peptide was also reduced by half serially.

Toxicity

Patients were observed 1h post-vaccination for immediate hypersensitivity and returned 48h later to have injection sites examined. Both local toxicity at the injection sites as well as systemic toxicity was evaluated in all patients for all inoculations. Toxicities were graded using National Cancer Institute Common Terminology Criteria for Adverse events v3.0 and reported on a 0-5 scale.

Peptides and Proteins

The AE37 peptide (Ac- LRMKGVGSPYVSRLLGICL-NH2) is a fusion of the li-Key peptide (LRMK) with the native HER2/neu peptide AE36 (aa776-790: GVGSPYVSRLLGICL) (19). To assess whether immune responses elicited in
vivo reflected reactivity against the native HER2/neu peptide, we evaluated immune responses against both AE36 and AE37. C.Albicans and Pokweed Mitogen (PWM) (Sigma, St Louis, USA) were used as positive controls for the in vitro immunogenicity assays. Peripheral blood mononuclear cells (PBMCs) in culture medium alone were used as a negative control.

**ELISPOT assay**

Blood was drawn from patients prior to treatment to establish a baseline (pre vac), then monthly before each inoculation, one month after the final (6th) vaccination (post vac) and six months after completion of vaccinations (long term). PBMCs were isolated by Ficoll gradient separation. Freshly isolated PBMCs were cultured in X-VIVO 15 medium (BioWhittaker™, Cambrex, Walkersville, MD) supplemented with 2% AB human serum (Sigma), with the individual peptides (10 µg/mL of AE36 or AE37) or controls (C.Albicans 1:50, PWM 2µg/mL) in pre-coated IFN-γ ELISPOT plates (MABTECH AB, Sweden), in quadruplicate at 2.5 x 10^5 cells/well. The plates were incubated at 37°C in a humidified 5% CO₂ incubator for 40h and developed as described by the manufacturer. Spots were enumerated using an ELISPOT analyzer (A.E.L.VIS GmbH, Hannover, Germany). Data are calculated in a way similar to that recently described (28). Briefly, data are presented as specific spots (i.e., experimental spots minus background spots, i.e. PBMCs in medium alone) per 10^6 PBMCs, or as a calculated 1/frequency of specific spots in 10^6 PBMCs. Patients were considered to have pre-existing immunity if, at baseline, the mean antigen-specific spots per well were statistically different (p<0.05) from background. Patients were considered to have increased response if the mean...
number of specific spots per well (MSPW, i.e. experimental number of spots per well minus the mean number of spots of the background wells at the corresponding cycle of vaccination) at a given vaccination round was greater than 2 standard deviations (SD) above the pre vac specific spots, remained the same if the MSPW was within 2 SD of the pre vac specific spots, and decreased if the MSPW was greater than 2 SD below the pre vac specific spots.

**Intracellular staining**

Cultures set up as above were harvested and recovered cells were stained with allophycocyanin (APC)-labeled anti-CD4, and with peridinin chlorophyll protein (PerCP)-labeled anti-CD3 antibodies (BD Biosciences, Franklin Lakes, NJ USA) at room temperature for 15 minutes. Cells were then washed, fixed, permeabilized and stained with fluorescein isothiocyanate (FITC)-labeled anti-IFN-γ (BD Biosciences) using BD Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences) according to manufacturer's protocol. Stained cells were analyzed by FACSCalibur and CellQuest software (BD Biosciences). Percentage of IFN-γ⁺ cells are presented corrected for background.

**Phenotypic characterization of Tregs**

PB from patients was collected pre vac, post vac, and at long term. Treg cells were detected in whole blood by a lyse-no-wash method. In brief, 50 μL of whole blood (with heparin or EDTA) were stained for 15 min at room temperature with pre-titrated combinations of anti-CD45-PerCP, CD4-APC, CD25-FITC and CD127-PE (all purchased from BD Biosciences). Red blood cells were lysed with 450 μL of ammonium chloride lysing solution and analyzed...
within an hour on a FACS Calibur. Tregs were defined by gating on low SSC CD45^+ CD4^+ lymphocytes expressing high CD25 (higher than the non CD4^+ lymphocytes) and negative/low for CD127 (29).

**Plasma HER extracellular domain and TGF-β determination**

Plasma samples were collected pre vac, post vac, and at long term. Commercially available immunoassay kits were used for measurements of TGF-β (human TGF-β1 instant ELISA, Bender MedSystems GmbH, Austria, Europe), and HER-2/neu extracellular domain (HER-ECD; Immuno 1, Bayer Diagnostics).

**Delayed-type hypersensitivity**

DTH reaction was performed before the vaccination regimen (pre vac DTH), post vac, and at long term. The DTH reaction was assessed with 100μg of AE36, which was injected intradermally at a site on the extremity opposite the one used for vaccination. DTH reactions, as well as dermal reactions to the vaccine, were measured in two dimensions at 48h using the sensitive ballpoint pen method and results are reported as an orthogonal mean (21). Patients must have an induration of >5mm post vac to be considered as having developed a positive DTH reaction (21).

**Statistical analysis**

GraphPad Prism version 4 software was utilized for the statistical analysis of data. Two-tailed Wilcoxon matched pairs test and Spearman correlation test at
95% Confidence Interval were used for statistical evaluation. Statistically significant differences were considered when p value was ≤ 0.05.
RESULTS

Patients

Thirty two patients with HER-2/neu+ PC were enrolled. Patient characteristics are given in Table 1. Twelve of the stage IV patients were metastatic with bone metastases. Three of the enrolled metastatic patients discontinued vaccinations (one patient at the second and two at the third cycle) at time of severe disease progression. Thus, 29 of the patients completed all vaccinations and could be evaluated immunologically. Treatment before vaccinations included: surgery, radiotherapy, chemotherapy (docetaxel) and androgen ablation (bicalutamide and LHRH agonist). During vaccinations, 19 patients did not receive any other treatment; 5 patients who had progressive disease received additional chemotherapy; the remainder received hormonal therapy alone or combined with radiotherapy.

Toxicity

Maximum local and systemic toxicities for all 32 patients are shown in Fig.1A. We observed no toxicities beyond grade 2. Predominant systemic symptoms were grade 0, with only 3 patients experiencing grade 1 fever during the first night which was easily manageable with paracetamol. Local toxicity was mild with most of the patients having grade 1 symptoms (pain at the injection site accompanied with mild swelling and itching) whereas grade 2 symptoms (large swelling with blisters at the injection site) were scored in 6 patients. Two patients required GM-CSF reduction, one after the first and one after the fourth vaccination. One additional patient required, initially (after the first vaccine) GM-
CSF reduction, but then, after the 2\textsuperscript{nd} vaccine, GM-CSF omission and finally at the 4\textsuperscript{th} vaccine peptide reduction.

**Dermal reactions**

Dermal reactions at each vaccination cycle (AE37 with GM-CSF) were determined 48h after immunization by measurement of induration. Already after the 1\textsuperscript{st} vaccination the vast majority of patients (25 of 29) developed dermal reactions which progressively increased after the 2\textsuperscript{nd} vaccination (27 of 29 patients), with all patients responding after the 3\textsuperscript{rd} vaccination cycle (Fig.1B).

**The AE37 vaccine induces IFN-γ-producing circulating T cells**

To study the effect of the AE37 vaccine on the immune system, PBMCs were isolated pre vac, during vaccinations, and they were analyzed for the presence of AE37-specific T cells by IFN-γ-based ELISPOT. The overall PBMC-derived IFN-γ response to AE37 and native AE36 peptide pre vac and during each round of vaccination is depicted in Figs.1C and 1D. The number of IFN-γ secreting cells in response to \textit{in vitro} stimulation with AE37 increased already after the 1\textsuperscript{st} round of vaccination (R1) and peaked two months later (i.e., after the 3\textsuperscript{rd} vaccine, R3) (Fig.1C). Patients’ PBMCs were also assessed \textit{in vitro} for IFN-γ response to the non-modified AE36 HER-2/neu(776-790) peptide, which is naturally processed and expressed on both, tumor cell lines and primary tumors from various types of cancers including prostate adenocarcinomas (18,19). The maximum AE36 peptide-specific immune responses were also observed after the 3\textsuperscript{rd} vaccination (Fig. 1D). Individual patient responses are depicted in Fig.1E. The median AE37-specific T-cell frequency pre vac was
The maximal response was a median frequency of one in 11,765 PBMCs ($p<0.0001$ compared to pre vac). Seventy-two percent of the patients (21 of 29) developed augmented immunity to AE37 peptide, seven patients (24%) did not increase, and one patient had a significant decrease upon immunization (Fig.1F). The median T-cell response to AE36 peptide pre vac was 1/83,333 (Fig.1E). During vaccinations, the median response to this peptide was increased up to a frequency of 1/19,231 ($p<0.0001$ compared to pre vac) (Fig.1E). Nineteen of twenty-nine patients (65.5%) developed augmented immunity, ten patients (34.5%) did not augment, and none had a significant decrease to AE36 with immunization (Fig.1F). Of note, 9 (31%) of 29 patients had pre-existent immunity to the native peptide and 5 of those significantly increased their responses during vaccinations ($p=0.0017$; Fig.1F).

The average IFN-$\gamma$ response to AE36 at long term assessment was comparable to average maximal response (Fig.1H) suggesting that the AE37 vaccine induces long-lasting systemic immunity.

**Both vaccine-specific and AE36-specific CD4$^+$ and CD8$^+$ T cells are induced by vaccination**

Development of immunity to either the AE37 vaccine peptide or to the native AE36 peptide was demonstrated by estimating the percentage of patients’ circulating T cells positively stained for intracellular IFN-$\gamma$ after exposure to the respective peptide. As shown in Fig.2, vaccination caused the successful induction of patients’ AE37-specific CD4$^+$ and CD8$^+$ T cell subsets (Figs.2A, 2D). We also observed a parallel increase of both T cell subsets responding to the native AE36 peptide (Figs.2B, 2E). The median maximal percentage of
CD4⁺ T cells specific for AE37 was 0.025 (Fig.2A) and 0.017 for AE36 (Fig.2B). Median pre-immunization values were less than 0.0001% for both AE37 and AE36. We also observed remarkable CD8⁺ T cell-mediated responses to AE37 (median maximal percent: 0.074%; p<0.0005 compared to pre vac) and to AE36 (median maximal percent: 0.085%; p<0.0001 compared to pre vac) (Figs.2D and 2E, respectively). Average maximal responses to AE36 for both subsets remained unchanged at long term assessment (Figs. 2C, 2F).

**Regulatory T cells in patients**

Tregs were defined as CD4⁺CD25⁺⁺CD127⁻⁻ cells (29). Immunization with the AE37 peptide and GM-CSF was associated with a statistically significant decrease of Treg frequency at long term (median: 4.89%; range: 1.30-9.00% in vaccinated vs 5.76%; range: 1.46-10.75% pre vac) (Fig.3A). Post vac Treg frequency was also reduced compared to pre vac (median: 5.18%; range: 2.12-10.46%), but without reaching statistical significance (p=0.4363).

**TGF-β and HER-2/neu extracellular domain (HER-ECD) levels in plasma**

TGF-β, a potent immunosuppressant of T cell immunity in cancer patients (30), has been demonstrated to be elevated in the serum of patients with prostate cancer (31, 32). The median level of plasma TGF-β pre vac was 9.34 ng/mL (range: 4.11-31.84 ng/mL). Post vac TGF-β levels, although reduced, did not reach statistical significance: median 8.46 ng/mL (range: 4.42-31.99 ng/mL; p=0.4043). However, at long term assessment a statistical significant reduction was achieved: 7.49 ng/mL(range: 2.90-24.23 ng/mL) (Fig.3B) Importantly, %
changes in long term, compared to pre vac, TGF-β plasma levels statistically correlated with the corresponding % changes in circulating Tregs (Fig.3C).

HER-ECD levels in serum have been reported to be elevated in prostate and breast cancer patients (33, 34). We show herein a significant decrease in plasma HER-ECD levels post vac (median: 5.9 ng/mL; range: 4.0-12.5) compared to pre vac (median: 9.2 ng/mL; range: 6.3-15.3 ng/mL) in our prostate cancer patients (Fig.3D).

**In vivo immunologic responses**

To determine more closely the effectiveness of the AE37 vaccine at inducing *in vivo* immunologic responses, we measured and compared DTH responses to the non-modified AE36 peptide pre vac, post vac, and at long term. Of the 29 patients who completed the vaccination series, 23 (79%) had significant post vac DTH reactions (>5mm) with a median post vac induration diameter of 11.5mm (range: 0.5-38.75mm) compared with a median of 0.0 mm (range : 0.0-8.0mm) at baseline (Fig.4). We did not observe any significant changes in long term DTH reactions (median 10.0mm; range: 0.5-45.0mm) compared to post vac DTH (Fig.4).

**Comparison of AE37-induced immunity among different patient groups**

Patients were retrospectively stratified for data analysis which might be useful for providing insight for patient selection proceeding to a phase II clinical trial. Retrospective stratification was done by staging [i.e., Stage II-III (non-metastatic patients) vs Stage IV (patients with advanced disease)], by response to testosterone lowering (i.e., CS vs CR), and by HER-2/neu expression (i.e.,
HER-2/neu low expressors with IHC 1+ and 2+ vs. HER-2/neu overexpressors with IHC 3+). As it can be seen from Figure 5, there was a tendency for better in vitro and in vivo immune responses to vaccination in the groups of patients with less progressed disease. The maximum increase of specific IFN-γ producing T cells in response to vaccination with AE37, detected by ELISPOT and expressed as mean fold increase from baseline levels, was higher in stage II-III (Fig 5A) and CS (Fig 5B) patients compared to stage IV and CR patients, respectively, although this difference was not statistically significant. Similarly, DTH responses to the vaccine, determined as the difference of post vac minus baseline DTH, were statistically significant lower in the stage IV patients (Fig 5D) and, also lower (although not statistically significant) in CR patients (Fig 5E). Interestingly, IFN-γ responses in patients with HER-2/neu overexpressing tumor at first diagnosis were highly reduced (with statistical significance) compared to patients exhibiting low HER-2/neu expression in their biopsies (Fig 5C). However, no statistically significant difference could be observed in the DTH responses according to HER-2/neu expression (Fig 5F).

Taking into consideration that such in vitro (IFN-γ) or in vivo (DTH) immunological responses might have been influenced by the tumor-induced suppressive milieu, we also evaluated the plasma TGF-β levels and the Treg frequencies before the initiation of vaccinations (baseline values) in the same groups of patients. Increased baseline TGF-β plasma concentrations were recorded in patients with progressed disease, i.e. stage IV (Fig 5G) and CR (Fig 5H), compared to stage II-III and CS patients, although not reaching statistical significance. No differences in pre vac Treg frequencies could be documented in any patient group (Figs 5J-5L).
DISCUSSION

We report here the results of a phase I trial evaluating the safety and immunological activity of a HER-2/neu hybrid peptide vaccine (AE37) in patients with castrate-sensitive and castrate-resistant, metastatic and non metastatic prostate cancer. This treatment was not associated with significant adverse events; as anticipated, patients experienced local injection site reactions, and a few experienced low-grade constitutional symptoms such as fever during the first night. The majority of patients developed T-cell (CD4⁺ and CD8⁺) immune responses specific for AE37 as well as for the native HER-2/neu(776-790) (AE36) peptide.

The Ii-Key technology has been designed to enhance presentation of antigenic peptides by promoting exchange of peptides in the MHC class II molecules at the cell surface (13). Accordingly, the AE37 hybrid peptide [Ii-Key/HER-2/neu(776-790)] is capable of directly charging the HLA-DR alleles with the epitope(s) present in HER-2/neu(776-790), thus inducing stronger immunologic responses in vitro and antitumor immunity in vivo than the native AE36 peptide. Indeed, we have demonstrated that AE37 elicits higher frequencies of IFN-γ⁺ CD4⁺ responder cells among cancer patients’ PBMC compared to AE36 and provided significantly stronger help to autologous CTL in lysing tumor cells both in vitro and in vivo (18, 19). Further, the AE37 hybrid vaccine was reported to elicit strong HER-2/neu-specific immune responses in a trial with breast cancer patients even in the absence of GM-CSF (21).
Peptide vaccine trials have focused predominately on stimulating CTLs, through the administration of 8-10mers binding on HLA class I alleles. Such vaccine-induced CTLs can directly kill tumor cells but lack long-lasting immunity (35). This AE37 peptide vaccine focuses on stimulating CD4+ T-helper cells with the goals of inducing immunologic memory and persistent stimulation of CTLs. Clinical studies utilizing class II peptide vaccines have been reported by Disis et al (36) in breast cancer. The Ii-Key modification of HER-2(776-790) has been used to vaccinate disease-free, node-negative breast cancer patients (21). In melanoma there are also class II peptide vaccines in clinical trials (37, 38). This is the first clinical trial using the Ii-Key modification in prostate cancer patients.

The HER-2/neu(776-790) peptide is promiscuously presented by many HLA-DR alleles (39). Such presentation theoretically could reflect one epitope which binds to many HLA-DR alleles and/or multiple closely overlapping, but slightly offset, HLA-DR epitopes. In either case, the AE37 vaccine appears to increase presentation and potency of T cell activation regardless of HLA allele. Whether the Ii-Key (LRMK) moiety actually confers additional MHC class II promiscuity to AE36 requires further investigation. Interestingly, our data from intracellular IFN-γ analyses detected potent responses mediated by patients’ CD8+ T cells suggesting that the AE37 vaccine, in addition to CD4+ T cells, also primes CD8+ T cells. Such responses were also detected in response to the native AE36 peptide suggesting that the Ii-Key moiety in AE37 enhances immunity directed against sequences with MHC class I binding motifs encompassed within HER-2/neu(776-790) (suggested by algorithm-prediction software). Moreover, IFN-γ production by CD8+ T cells in response to AE37 may also be triggered by MHC “neo-determinants” generated by the linkage if Ii-Key to AE36. Although this has
to be confirmed, our data suggest that this might be the case given the higher frequencies of responders’ IFN-γ+ cells with AE37 as compared to AE36. The efficacy of our vaccine formulation to induce a specific T-cell response against AE37 and/or AE36 has been confirmed by the high number of patients with augmented immune responses. Such responses usually peaked by the 3rd round of vaccination and then declined. One possibility for this could be that after a number of vaccinations subsets of the vaccine-induced T cells acquire different migratory or homing characteristics and they selectively accumulate to various body compartments, including lymph nodes, bone marrow and/or tumor tissue (40).

Stimulating CD4+ T-helper cells is essential for inducing long-term immunity. In most of our vaccinated patients, we could detect specific immunity to AE36 both in vitro (IFN-γ) and in vivo (DTH) even 6 months after completion of the vaccination series. However, vaccinating with MHC class II-restricted peptides might additionally activate Tregs, which are already elevated in prostate cancer patients (41-43). By monitoring circulating Tregs in our AE37 trial, we detected a reduction in the frequency of Tregs post vaccination which was even more pronounced at long-term assessment. Furthermore, Treg frequency reduction correlated with a decrease in TGF-β plasma levels, thus indicating that vaccination with AE37 may effectively modulate the immunosuppressive milieu induced by the tumor. The fact that Tregs, as well as TGF-β, were found to be more reduced six months after completion of vaccinations (long term values) compared to post vaccination is indicative of ongoing immune responses triggered by the vaccine, probably not solely against the targeted epitope (AE36), but against a broad tumor-associated specificities (epitope spreading).
This has also been reported when vaccinating metastatic breast cancer patients with MHC class II restricted HER-2/neu peptides (28).

HER-ECD serum levels have been demonstrated to be elevated in prostate cancer patients and shown to be an independent prognostic factor associated with high risk of biochemical recurrence (33). For this reason, it was of interest to determine plasma HER-ECD levels at enrolment and after completion of vaccinations. Overall, a significant vaccine-induced decrease in HER-ECD plasma levels was observed. Because of the limited number of patients, however, we could not detect any statistical difference among patients with different clinical characteristics. Further clinical studies will be required to clarify the reason for this decrease.

By retrospectively analyzing our patients according to their pre-enrolment characteristics, i.e. stage, sensitivity to biochemical castration or HER-2/neu expression, we have detected a correlation between the extent of the disease and the in vitro and in vivo vaccine-induced immune responses against AE36. Patients with advanced disease, stage IV and/or CR, exhibited decreased circulating IFN-γ producing cells and lower post vac DTH responses. It has been previously documented (44) that cancer progression leads to tumor-mediated local and systemic immunosuppression resulting in compromised antitumor immunity. Indeed, we found in the present study that pre-vaccination baseline plasma TGF-β levels were increased in patients with advanced disease (stage IV and/or CR), at least partially accounting for a hostile milieu in these groups leading to decreased AE37 vaccine-induced immune responses.

In contrast, similar percentages of peripheral blood Tregs were detected among the various groups. Our data are in line with those of Yokokawa et al (42) who
found no significant differences in Treg populations in peripheral blood of prostate cancer patients with localized or advanced disease, yet showed that Tregs from metastatic patients were more suppressive.

It has been previously reported that breast cancer patients with low HER-2/neu (IHC 1+) expressing tumors responded better to the E75 vaccine than those with HER-2/neu IHC 2+ and 3+, suggesting an element of immunologic tolerance in the high HER-2/neu expressors (45). In the present study we also found significantly reduced INF-γ producing cells and lower (although not statistically significant) DTH responses in patients with high (IHC 3+) HER-2/neu expression compared to HER-2/neu IHC 1+ and 2+.

Given the small number of vaccinated patients, their heterogeneity and the various types of treatments, it was difficult to determine whether an association exists between the development of an immune response and clinical response. Thus, we could not detect a significant association between vaccine-specific immunological responses and PSA levels or OS/PFS (data not shown). Furthermore, most patients (13/29, of which 12 did not receive any therapy during vaccinations) when entering the study had negligible PSA values which remained low (below 0.2 ng/mL) throughout the relatively short vaccination period. Obviously, this could be a result (i) of the previous therapies, (ii) of the vaccine or (iii) of a combination thereof.

In conclusion, use of the AE37 immunotherapeutic vaccine is a novel strategy for managing patients with prostate cancer. This clinical trial demonstrates that the vaccine is safe and immunologically active. However, proof of clinical benefit will require a phase II trial in a homogeneous group of patients with less extensive disease, including disease-free patients at high risk of recurrence.
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We are indebted to our patients for their voluntary participation onto this study. We thank Dr. Angela Ferderigou, head of the Biochemistry Department of Saint Savas Cancer Hospital for providing us with patients’ PSA data and Ms Joanne Calogeropoulou for excellent technical assistance.
FIGURE LEGENDS

**Figure 1.** Toxicity, dermal reactions, and immunity to AE37. (A) Maximum local and systemic toxicities observed during vaccinations (graded according to common terminology criteria). (B) Dermal reactions (orthogonal mean±SEM) were recorded 48h post vaccination throughout the vaccination series. **: p=0.0013 and ***: p<0.0001. IFN-γ ELISPOT responses to AE37 (C) or to AE36 (D) during vaccinations with AE37. Specific spots (mean ±SEM) are shown at baseline (R0) and 1 month after each vaccination (R1 to R6). (E) Individual pre vac and maximal (max) ELISPOT IFN-γ responses obtained during vaccination. Horizontal bars: median values. (F) Percent AE37 and AE36 specific immunity after vaccination. Gray: increased; hatched: unchanged; black: decreased. (G) Pre vac and maximal ELISPOT IFN-γ responses in patients with pre-existent immunity to AE36. (H) Durable immunity to AE36 induced by the AE37 vaccine: mean±SEM. IFN-γ ELISPOT responses to AE36 at long term did not differ compared to maximal responses.

**Figure 2.** Increased numbers of both, CD4+ and CD8+ IFN-γ producing T cells after vaccination. (A, D) Individual responses to AE37 and (B, E) to AE36. Vaccine-induced AE36-specific CD4+IFN-γ+ (C) or CD8+IFN-γ+ (F) subsets during vaccinations (maximal responses) and at long term.

**Figure 3.** Biological and immunological parameters pre vac, post vac and at long term. (A) Circulating Tregs; serum levels of (B) TGF-β and (D) HER-ECD.
(C) Correlation between % change in TGF-β and % change in Treg frequency at long term compared to pre-vac values. Horizontal bars: median values.

Figure 4. Delayed-type hypersensitivity pre vac, post vac and at long term. Box and whiskers (5-95% percentile) plot. Horizontal bars: median values. Vertical bars: range.

Figure 5. Comparison of immune parameters in vaccinated patients classified according to staging (Stage II-III vs Stage IV), sensitivity or resistance to testosterone lowering (CS vs CR, respectively) and the levels of their tumors' HER-2/neu expression (HER1^+2^+ vs HER3^+). In (A)-(C) results represent max specific spots divided by pre vac specific spots. In (D)-(F) results represent DTH post vac minus DTH pre vac. In (G)-(I) plasma TGF-β concentrations are depicted and in (J)-(L) % circulating Tregs. Where not indicated, intragroup comparisons were not statistically significant. Total: all patients. Bars represent mean values ±SEM.
### Table 1

**Patient characteristics**

<table>
<thead>
<tr>
<th></th>
<th>%</th>
<th>No of Patients</th>
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<tr>
<td><strong>Age, median (range)</strong></td>
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<tr>
<td><strong>Race</strong></td>
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<tr>
<td>Caucasian</td>
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<td>32</td>
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<tr>
<td><strong>Gleason score</strong></td>
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<td>Low (3-6)</td>
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<td>Intermediate (7)</td>
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<td>High (8-10)</td>
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<td>Castrate-resistant</td>
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<td><strong>PSA</strong></td>
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<td>PSA &lt; 0.2ng/ml</td>
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<td>PSA 0.2-10ng/ml</td>
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<td>PSA &gt;10ng/ml</td>
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<tr>
<td>All median (range)</td>
<td>0.86 (0.01-367) ng/mL</td>
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</tr>
</tbody>
</table>

(*) Staging according to AJCC
Figure 2

A

AE37

B

AE36

C

AE36

pre vac max

pre vac max long term

D

AE37

E

AE36

F

AE36

pre vac max long term

% CD8+ IFN-γ

% CD8+ IFN-γ

% CD8+ IFN-γ

% CD8+ IFN-γ

% CD8+ IFN-γ

% CD8+ IFN-γ

% CD8+ IFN-γ
Clinical Cancer Research

Results From a Phase I Clinical Study of the Novel Il-Key/HER-2/neu(776-790) Hybrid Peptide Vaccine in Patients with Prostate Cancer

Sonia A Perez, Kallinteris L Nikoletta, Bisias Stratos, et al.

Clin Cancer Res  Published OnlineFirst May 13, 2010.

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