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Serum *Apolipoprotein E* and Other Inflammatory Markers Can Identify Non-Responding Patients to a Dendritic Cell Vaccine[1,2]

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Abstract

BACKGROUND: Despite the majority of patients do not gain any benefit from dendritic cells (DC) vaccines, this approach has occasionally given rise to dramatic responses in melanoma. Biomarkers are crucial to identify which patients are more likely to respond. We looked for correlations between pre- or post- vaccination biomarkers and clinical outcomes to DC therapy in a cohort of patients with stage IV melanoma receiving a vaccine with autologous *ex-vivo* expanded DCs pulsed with allogeneic tumor cell lysate. *METHODS:* Serial serum samples were collected at baseline, week 4 and 12 and they were analyzed for a panel of different inflammatory markers using cytometric bead array technology and ELISA. *RESULTS:* Twenty-one patients were evaluable for response. Patients were separated into responders and non-responders based on clinical benefit. Responders were defined as patients who achieved a complete response, partial response or stable disease the latter lasting for at least 6 months. Responders (N = 9) showed a significantly longer Progression-free Survival (PFS; HR 0.23; 95% CI 0.08–062; $P < .001$) and Overall Survival (OS; HR 0.22; 95% CI 0.08–0.59; $P < .001$). The clinical non-responder phenotype correlated with an elevated pre-vaccination level of cytokines associated with inflammation compared to clinical responders (Apolipoprotein C111; IL-12 p40; MiP1α; Stem Cell Factor and TNFα). Apolipoprotein E (ApoE) was also significantly elevated in the pre-vaccine sera of the clinically non-responding group and in addition it was found to correlate with outcomes. Patients with increased levels of ApoE had a significantly shorter PFS (HR 3.02; 95% CI 1.09–8.35; $P = .015$) and OS (HR 2.40; 95% CI 0.9–6.3; $P = .034$). *CONCLUSION:* Our findings support the notion that treating the inflammatory background may have an impact on clinical outcome for patients receiving immunotherapy. A larger study is needed to confirm the significance of ApoE as a predictive biomarker for response to DC vaccines.

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Introduction

Autologous dendritic cell (DC) vaccines have been used in the past 25 years for the treatment of cancer with mixed outcomes [1]. Although good clinical responses have been reported and a DC-based vaccine has even been granted approval by the FDA for the use in advanced prostate cancer [2], the vast majority of the studies resulted in objective response rates of less than 10% with the clinical benefit generally limited to a period of about 3 months.

A number of different reasons have been suggested to explain why DC vaccines do not produce better clinical results. Among these reasons are (1) the complexity of the isolation and differentiation/

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maturation procedures involved in vaccine preparation (2) the variability of the antigen loading and DC maturation protocols (3) the source and type of antigen used and (4) the presence of an inflammatory immunosuppressive tumor microenvironment that cannot be overcome by the DC-based vaccine.

In this respect melanoma patients with elevated inflammatory markers such as raised LDH, lymphocyte/neutrophil ratio, C-reactive protein [3] and inflammatory cytokines such as IL-6 and IL-1ra have a generally poor prognosis and tend not to respond to treatment [4,5].

A few years ago we completed an early phase study with autologous *ex-vivo* expanded DCs pulsed with allogeneic tumor cell lysate in patients with metastatic melanoma to assess the safety and the feasibility of this approach. We collected serial serum samples from the patients on study to evaluate panel inflammatory markers and we report the results here.

Materials and Methods

Clinical Trial

This was a phase I/II study of autologous *ex-vivo* expanded DCs pulsed with allogeneic tumor cell lysate in patients with unresectable metastatic melanoma. The study was approved by the Local Ethics Committee at St George's Hospital Medical School (Ethics Committee reference number: 03.0285) and the endpoints were primarily safety and feasibility. Patients with stage IV melanoma (either treatment naïve or pre-treated) with an ECOG ps 0–1, no prior therapy for 4 weeks and life expectancy greater than 3 months were considered eligible Exclusion criteria included concurrent treatment, cerebral metastases other than those stable after 3 months of treatment, abnormal renal (Creatinine >140 μmol/L) or liver function test (Bilirubin >1.5 × normal limit or AST/ALT/ Alk Phos >2 × upper limit of normal), excessive tumor burden (at the physicians' discretion).

All patients received 1-3 × 10^6 tumor lysate-pulsed DCs intradermally at 2 weeks interval for 12 weeks and eventually monthly thereafter until radiological or clinical progression. In addition, low dose IL-2 (6MIU units daily subcutaneously for 3 days) was given after every vaccination. Disease was re-assessed every 3 months with standard imaging and responses evaluated as per RECIST 1.1. Cryopreserved vaccines were recovered from storage in liquid nitrogen by thawing in a 37°C water bath over 100 s, before being re-cultured for 24 h.

Table 1. Complete list of analytes in RBM human MAP panel, used for determination of serum proteins

Alpha Fetoprotein	Erythropoietin	IL-5	Myoglobin
Alpha-1 Antitrypsin	Factor VII	IL-6	PAI-1
α-2 Macroglobulin	FABP	IL-7	PAP
Adiponectin	Ferritin	IL-8	PAPP-A
Apolipoprotein-A-1	FGF-basic	IL-10	SGOT
Apolipoprotein-CIII	Fibrinogen	IL-12 p40	SHBG
Apolipoprotein-H	G-CSF	IL-12 p70	PSA, Free
BDNF	GST	IL-13	RANTES
β-2 Microglobulin	GM-CSF	IL-15	Serum Amyloid P
C Reactive Protein	Growth Hormone	IL-16	Stem Cell Factor
Calcitonin	Haptoglobin	IL-18	TBG
Cancer Antigen 19–9	ICAM-1	Insulin	Thrombopoietin
Cancer Antigen 125	IFN-gamma	Leptin	TIMP-1
CEA	IgA	Lipoprotein (a)	Tissue Factor
CD 40	IgE	Lymphotactin	TNF-α
CD40 Ligand	IGF-1	MCP-1	TNF-β
Complement 3	IgM	MDC	TNF RII
CK-MB	IL-1α	MIP-1α	TSH
EGF	IL-1β	MIP-1β	VCAM-1
ENA-78	IL-1ra	MMP-2	VEGF
Endothelin-1	IL-2	MMP-3	vWF
ENRAGE	IL-3	MMP-9	
Eotaxin	IL-4	Myeloperoxidase	

Table 2. Patients` Characteristics

Age/ Gender	Stage AJCC v.7	ECOG PS	BOR	Treatment-naive	PFS (months)	OS (months)
64 male	IVb	0	SD	No	7	18 months
50 male	IVc	0	CR	Yes	85 months - Alive	85 months - Alive
75 male	IVa	0	PR	Yes	24	80 months – Alive
38 male	IVc	1	SD	No	6	38 months
48 male	IVc	0	SD	Yes	6	18 months
60 male	IVc	0	SD	No	6	10 months
45 male	IVc	0	PR	Yes	16	75 months
50 male	IVb	0	SD	No	7	14 months
30 male	IVc	0	CR	No	81 months - Alive	81 months - Alive
53 male	IVb	0	SD	No	4 months	16 months
73 male	IVb	1	SD	Yes	4 months	15 months
60 male	IVa	0	PD	Yes	3 months	11 months
55 male	IVa	0	PD	No	3 months	10 months
67 male	IVa	0	PD	No	3 months	4 months
49 female	IVa	0	PD	No	3 months	10 months
49 male	IVc	1	PD	Yes	3 months	7 months
70 male	IVa	0	PD	No	3 months	7 months
74 male	IVa	0	PD	Yes	3 months	5 months
70 male	IVc	1	PD	Yes	3 months	3 months
60 male	IVc	0	PD	No	5 months	5 months
54 male	IVb	0	PD	No	4 months	4 months
63 male	IVa	1	NE	Yes	NE	NE

BOR: Best Overall Response, SD: Stable Disease, PR Partial Response, CR Complete Response, PD Progressive Disease; PFS: Progression-free survival, OS: Overall Survival; NE; not-evaluable.

Serum Collection

Samples were collected up to 72 hours prior to the first vaccine and at week 4 and 12 on study prior to administration of the vaccine. Ten milliliters of blood was collected into EDTA tubes by venepuncture. Tubes were centrifuged at 2000G for 10 minutes and serum was subsequently aspirated from the cell pellet. Serum was frozen at -70°C within 1 minute of separation and stored until use.

Dendritic Cells Isolation and Differentiation

One unit of peripheral blood was taken from study participants by apheresis, centrifuged to isolate the cell pellet and labeled with Clinimax CD14 beads according to the manufacturer's instructions (Miltenyi). Cells were isolated using the Enrichment 1.1 protocol on the Clinimax apparatus. Isolated CD14 cells were washed in phosphate buffered saline (PBS) and then differentiated using IL-4 and GM-CSF (Peprotech and Leukomax respectively). CD14+ cells were cultured in 25 ml of RPMI1640 supplemented with 5% human AB serum (Gibco) in T175 flasks at 1-2 × 10^6 cells ml^{-1} with GM-CSF (100ngml^{-1}) and IL-4 (50ngml^{-1}) for 7 days. Cytokines were refreshed at day 2 and 4. On day 7, cells were harvested, washed and counted.

Tumor Lysate Preparation and DC Loading

Generation of monocyte-derived DCs, pulsing with tumor cell line derived lysates and freeze/thaw maturation process have been detailed elsewhere [6]. Melanoma tumor lines (KM, MJT3 and NF) were grown as previously described [7]. Melanoma cells were irradiated with 150Gy and then lysed by repeated freeze/thaw cycles. Lysates were assessed for residual cell viability using Trypan Blue staining and were passed through 0.2 μm filters to remove cell debris. Protein concentration was determined by Bio-Rad protein assay kit. Tumor

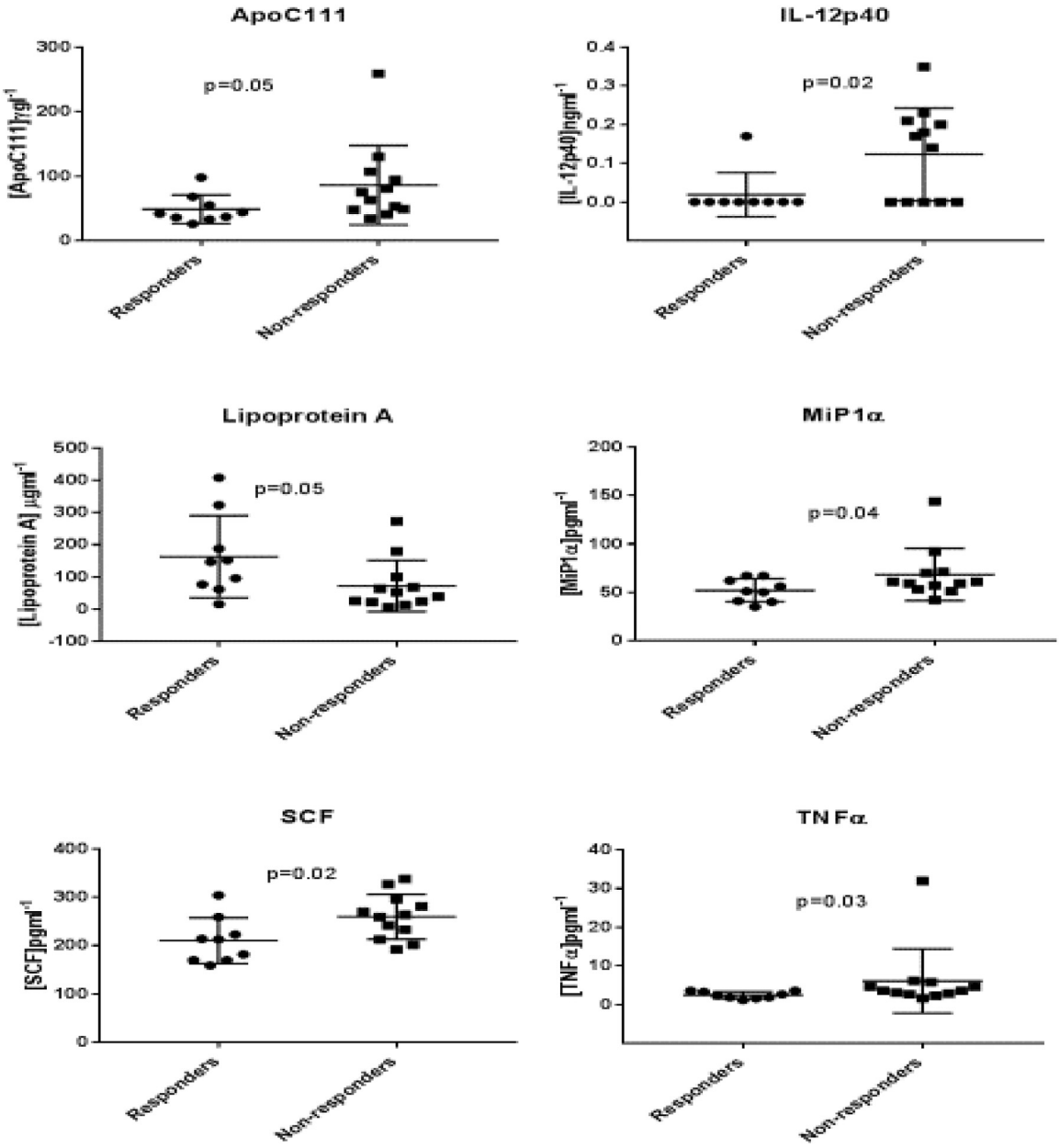

Figure 1. Pre-vaccination serum protein differences between responders (n = 9) and non- responders (n = 12). Figure shows mean and standard deviation. p values are student's t-test for normal data and Mann–Whitney for non-normal data.

lysate was stored at −80°C until used. On day 7 DCs were re-plated at $3x10^6$ cell ml^{-1}, tumor lysate was added to a final concentration of $100\mu gml^{-1}$ and cells cultured for 2 hours in RPMI 1640, supplemented with 5% human AB serum (Gibco) at 37°C. IL-4 and GM-CSF were added for a further 2 hours and then cells were harvested, aliquoted and stored in liquid nitrogen for subsequent use.

Serum Biomarker Analysis

Cytometric bead array was performed by Rules Based Medicine (RBM; see website for details: www.rulesbasedmedicine.com/) using their basic Human Multi-Analyte Profile (MAP) array. Briefly, a multiplex bead array system was employed to determine the levels of a number of markers, including cytokines and chemokines, present in the serum of patients. A complete list of analytes is shown in Table 1.

The concentration of Apolipoprotein E3 (ApoE3) in sera was measured with an in-house ELISA. Ninety-six-well NUNC maxisorp microtitre plates were coated with 50 µl/well of 1 µg/ml rat anti-human ApoE3 (MAB41441, R & D Systems, UK) in PBS overnight at 4°C. The plates were washed 4 times with PBS containing 0.05% Tween (PBS-T) and then blocked with 300 µl/well PBS containing 1% bovine serum albumin (BSA, Sigma, UK) for 1 hour at room temperature. After 4 washes with PBS-T, 50 µl/well human recombinant ApoE3 standard (4144-AE, R & D Systems, UK) diluted serially from 1000 to 0.1 ng/ml and human serum samples diluted 1/1000 (all in PBS containing 1% BSA) were added to the plates in triplicate and incubated for 2 hours at room temperature. After 4 washes with PBS-T, 50 µl/well goat anti-human Apo E (AF4144, R & D Systems, UK) diluted to 1 µg/ml was added and incubated overnight at 4°C. After 4 washes with PBS-T, 50 µl/well rabbit anti-goat IgG-HRP (R & D Systems, UK) at 1/1000 was added and incubated for 30 minutes at room temperature. The plates were washed a further 4 times with PBS-T and 2 times with 0.05 M

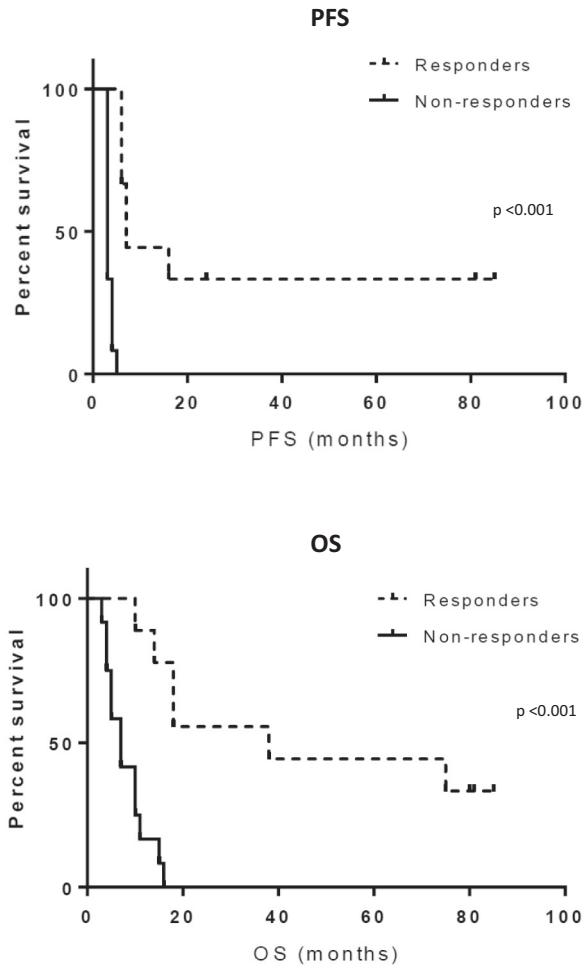

Figure 2. Kaplan–Meier estimates of PFS and OS of responders (N = 9) vs. non-responders (N = 12).

phosphate–citrate-buffer (pH 5.0) before developing with 100 µg/ml 3,3′,5,5′-tetramethylbenzadine dihydrochloride in phosphate–citrate-buffer (pH 5.0) containing 0.006% H_2O_2. Finally, the reaction was stopped with 12.5 µl/well 1 N H_2SO_4 and the optical densities of the wells were read at 450 nm with an ELx800 microplate reader (Bio-Tek, UK). The coefficients of variance for interplate and interday variation of this ELISA were 4.2% and 4.6% respectively. The levels of serum MiP-1α and ICAM-1 were measured using commercial kits (DY270 DuoSet and DY720 DuoSet respectively, R & D Systems, UK) according to the manufacturer's instructions.

Statistical analysis

The data were analyzed using Prism research software with Student's *t*-test or Mann–Whitney *U* test, dependent on normality. A *P* value <0.05 was considered statistically significant.

Progression-free survival (PFS) was calculated from time of enrolment until disease progression or death/last follow-up. Overall Survival (OS) was calculated from time of enrolment to death or last follow-up. Kaplan–Meier analysis was used to estimate PFS and OS. The log rank test assessed differences in progression or survival in patients with different immunological or clinical parameters.

Results

Clinical Outcomes

Twenty-two patients (21 male and one female) were enrolled in the study (Table 2). Patients received a median of 6 vaccines (range 1–12) with no significant local or systemic toxicity. Twenty-one patients were evaluable for response. One patient discontinued treatment before radiological disease re-assessment. Four out of the 21 (19%) patients evaluable experienced an objective response as per RECIST 1.1 criteria. Two patients achieved a complete response (CR) and they are still alive at time of writing with an estimated overall survival of about 80 months for both. Seven patients had a stability of disease (SD). For five of these patients the disease remained stable for a period of 6 months or more for a total calculated clinical benefit (CB = CR + PR + SD ≥ 6 months) of 43% (9/21).

Upon closure of the trial, patients were retrospectively stratified into non-responder and responder based on the CB. Responders showed a significantly longer PFS (HR 0.23; 95% CI 0.08–062; $P <$ .001) and OS (HR 0.22; 95% CI 0.08–0.59; $P <$ .001; Figure 2). At the time of writing, 0/12 of the non-responders have survived however 3/9 of the responder group are alive.

Serum Protein Analysis: Pre-Vaccination Differences in Responders and Non-Responders

Pre-vaccination serum samples from the 12 clinically non-responding patients and 9 responding patients were quantified by means of a multiplex approach. We have analyzed 90 different serum proteins (Table 1) and results from the two different cohorts were compared.

We detected significant pre-vaccination differences between the non-responder population and the responder population for six proteins (Figure 1). Apolipoprotein C111 (86.2 μgml^{-1} vs. 48.8 μgml^{-1}; P = .05), IL-12 p40 (0.12 $ngml^{-1}$ vs. 0.02 $ngml^{-1}$; P = .02), MiP1α (68.4 $pgml^{-1}$ vs. 52.1 $pgml^{-1}$; P = .04), stem cell factor (259.8$pgml^{-1}$ vs. 210.4$pgml^{-1}$; P = .02) and TNFα (6.1 $pgml^{-1}$ vs. 2.4 $pgml^{-1}$; P = .03) levels were significantly increased in the non-responder group compared to the responders. In contrast to the elevation of these pro-inflammatory mediators seen in the non-responsive group of patients, a significant decrease in Lipoprotein A in the non-responders compared to responders (72.3 μgml^{-1} vs. 163.2 μgml^{-1}; P = .05) was observed. (See Fig. 4.)

No significant differences were observed between responders and non-responders for serum samples obtained at weeks 4 and 12. No significant changes were observed between baseline and samples at week 4 and 12.

Validation of Markers Using ELISA and Predictive Techniques

Since the low study numbers preclude the use of type 1 error correction in the cytometric bead data, we sought to identify additional methods to validate these data. Initially we used a powerful literature search software (Pathway studio) to develop a network of connectivity between the potential markers identified and then we attempted to find additional molecules that would fit into this network. These approaches led to the identification of ApoE as a putative additional marker. ApoE was quantified by ELISA and, as predicted, a significant difference between the non-responder and responder patients was observed (129.6 µg/ml responders vs. 201.5 µg/ml non-responders; P = .005; Figure 3). MiP1α and ICAM-1 were added as controls and ELISA data confirmed the original observations (MiP1α 42.0 pg/ml responders vs. 134.1 pg/ml non-responders; P = .03; ICAM 258.6 ng/ml responders vs. 286.9

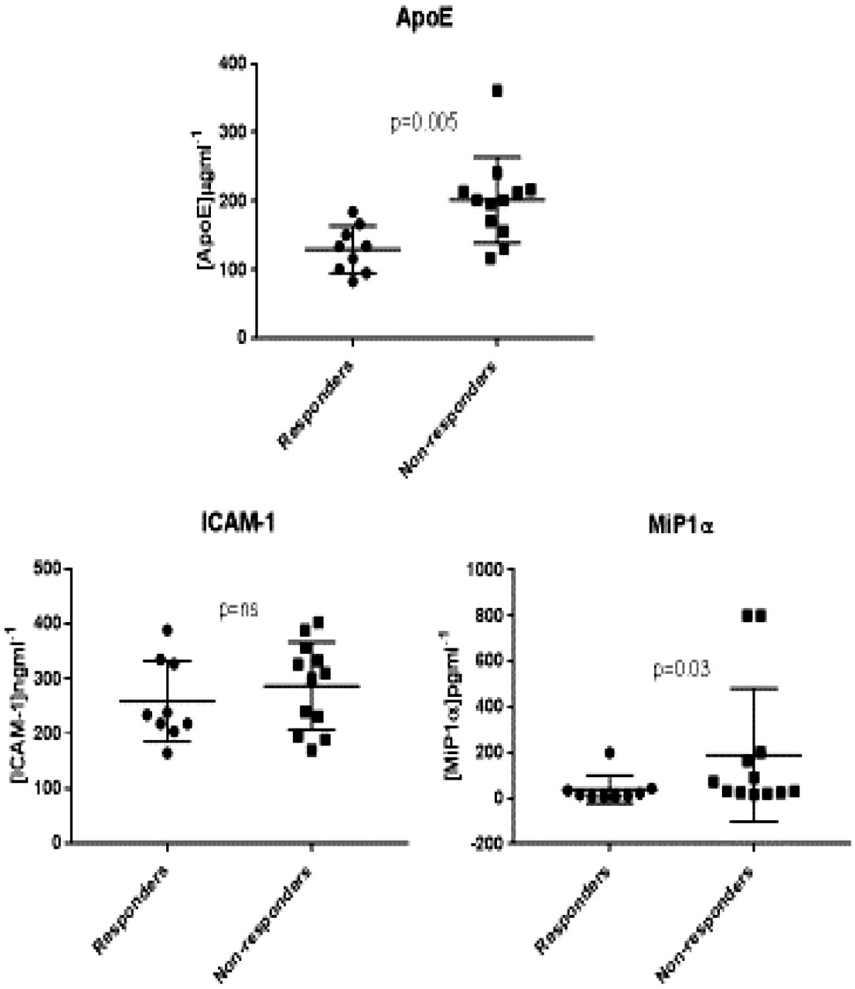

Figure 3. Confirmatory serum analyte quantification determined by ELISA and Apolipoprotein E. Statistical tests are non-paired parametric (Student's *t*-test) or non-parametric (Mann–Whitney *U*), dependent on normality of data.

ng/ml non-responders; $P > .05$; Figure 3). It must be noted that the ELISA and RBM microarray results differ in magnitude suggesting differences in the sensitivity of the two assays.

The mean value of ApoE observed in this cohort of subjects was selected to categorize patients as higher or lower ApoE serum level. Patients with levels of ApoE above the average had a significantly shorter PFS (HR 3.02; 95% CI 1.09–8.35; $P = .015$) and OS (HR 2.40; 95% CI 0.9–6.3; $P = .034$). No differences were observed for MiP1α.

Neutrophil/lymphocyte Ratio and LDH

No significant differences were observed in the neutrophil/lymphocyte ration and LDH levels over time nor between responders and non-responders (data not shown).

Discussion

Despite that the majority of patients do not gain any benefit from DC vaccines, this approach has occasionally given rise to dramatic responses in melanoma. Biomarkers are crucial to identify which patients are more likely to respond to DC vaccines. We looked for correlations between pre- or post- vaccination biomarkers and clinical outcomes to DC therapy in a cohort of patients with stage IV melanoma receiving an autologous DC vaccine. We have identified a general inflammatory marker profile and we validated the data using literature-searching software to predict for additional markers, which would be expected to be elevated if the original data is correct. We thus identified ApoE as an additional biomarker of non-response and we have subsequently confirmed this by ELISA.

ApoE has been the focus of intense interest with regard to immunomodulation as well as cancer for more than three decades. Despite that, there is still a lack of consensus on the role of ApoE in various cancers, overexpression of ApoE resulting in elevated levels in the serum, plasma or urine is associated with a poor prognosis or advanced stage in human cancers, including lung [8–10], gastric [11,12], ovarian [13,14] and bladder cancer [15,16]. On the contrary, ApoE was also shown to act as a direct metastasis-suppressive factor in melanoma [17], identifying ApoE as a potential biomarker for assessing tumor stage, metastasis, prognosis or response to treatment. Van den Elzen et al. proposed a pro-inflammatory role of ApoE, showing ApoE involved in the presentation of CD1a-loaded lipid antigens by antigen presenting cells (APC) for recognition by natural killer T (NKT) cells, which in turn respond by secreting cytokines (e.g. IFN-γ) and initiating an immune response [18]. A pro-inflammatory role of ApoE could also be due to its association with cell lipid homeostasis. ApoE possess lipophilic properties, potentially removing cholesterol and other lipids from the cell membrane and consequently initiating the recruitment of TLRs to lipid drafts as a

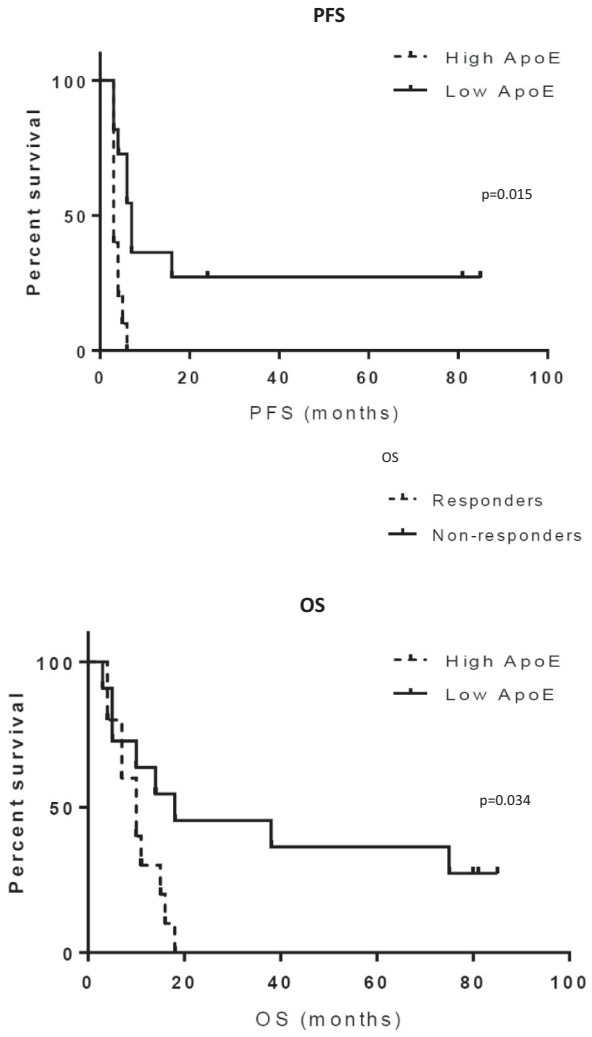

Figure 4. Kaplan–Meier estimates of PFS and OS of patients with higher (N = 10) vs. lower serum level (N = 11) of ApoE.

possible signal of cell damage [19]. As a result, transcriptional factors, such as NF-κB and interferon regulatory factors (IRFs) are activated and contribute to the production of pro-inflammatory cytokines [20,21]. Combined with additional markers, such as those identified in this study, measurement of ApoE could identify inappropriate inflammation in patients prior to treatment and thus identify patients who are most unlikely to benefit from DC immunotherapy.

Inflammation in cancer may be caused by tumor invasion and this perturbation of homeostasis could generate a sterile inflammatory response [22]. Intuitively inflammation would seem to be prerequisite for activation of the immune response and the generation of an anti-tumor outcome. However, chronic inflammation has a negative correlation with cancer outcomes [23,24]. The immune response status at the time of diagnosis may have a bigger impact on prognosis than the typical staging procedures irrespective of other treatments given [25].

One possible mechanism for the influence of inflammation on cancer is the shift between a desirable Th1 (cell mediated immunity associated) response and a non-effective Th2 (humoural immunity associated) response [26]. If this were the case, then one clinical possibility would be to pre-treat patients about to undergo immunotherapy with an anti-inflammatory protocol.

It is interesting to note that there are several reports, which have observed an improved response to vaccination in mice when they have been pre-treated with known anti-inflammatories. At present, the strongest evidence available that confirms pre-treating is beneficial has been shown with Lenalidomide [27,28]. This was subsequently confirmed in humans with multiple myeloma patients who responded to Prevnar after they had been pre-treated with Lenalidomide [29]. Although Lenalidomide has been reported as having a co-stimulatory function, it is also a strong inhibitor of inflammation through the Cox-2 pathways [30]. Hence, a pre-immunotherapy course of anti-inflammatory treatment might render the potentially unresponsive patient clinically responsive. In this study we have described a panel of potential inflammatory markers that can help to identify patients less likely to response to a DC-vaccine. Other groups have looked at larger cohorts of patients treated with PD-1 inhibitors or high dose IL-2 and they have detected similar biomarkers. In keeping with our findings, they are present prior to treatment and rather than as a result of treatment, they are elevated in non-responders and include many markers of chronic inflammation as described here [31,32].

Conclusions

In summary, our findings support the notion that treating the inflammatory background may have an impact on clinical outcome for patients receiving immunotherapy. A larger study is needed to confirm the significance of ApoE as a predictive biomarker for response to DC vaccines.

Declarations

Ethics Approval and Consent to Participate

The study was approved by the Local Ethics Committee at St George's Hospital Medical School (Ethics Committee reference number: 03.0285). Study participants signed informed consent prior to study participation.

Consent for Publication

This manuscript does not contain individual data presented in a manner that could identify them.

Availability of Data and Materials

Data can be released in conjunction with study participant signed consent form. The datasets analyzed during the current study are not publicly available given the restrictions arising from the signed consent.

Author Contributions

AGD was the PI of the clinical trial and supervised the whole project. Experiments were designed by MBS and carried out by EK and AG under the supervision of MBS, GC and JC. The ApoE ELISA was developed by KBS. HL and DG acquired the clinical data. Analysis of the clinical data was carried out by AF and AGD. The paper was written by MBS, AF and AGD with contributions from HL, AG and JC.

All Authors read and approved the manuscript.

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