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ORIGINAL ARTICLE

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Clinical validation of circulating GDF15/MIC-1 as a marker of response to docetaxel and survival in men with metastatic castration-resistant prostate cancer

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Abstract

Background: Elevated circulating growth differentiation factor (GDF15/MIC-1), interleukin 4 (IL4), and IL6 levels were associated with resistance to docetaxel in an exploratory cohort of men with metastatic castration-resistant prostate cancer (mCRPC). This study aimed to establish level 2 evidence of cytokine biomarker utility in mCRPC.

Methods: IntVal: Plasma samples at baseline (BL) and Day 21 docetaxel ($n = 120$). ExtVal: Serum samples at BL and Day 42 of docetaxel ($n = 430$). IL4, IL6, and GDF15 levels were measured by ELISA. Monocytes and dendritic cells were treated with 10% plasma from men with high or low GDF15 or recombinant GDF15.

Results: IntVal: Higher GDF15 levels at BL and Day 21 were associated with shorter overall survival (OS) (BL; $p = 0.03$ and Day 21; $p = 0.004$). IL4 and IL6 were not

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associated with outcomes. ExtVal: Higher GDF15 levels at BL and Day 42 predicted shorter OS (BL; $p < 0.0001$ and Day 42; $p < 0.0001$). Plasma from men with high GDF15 caused an increase in CD86 expression on monocytes ($p = 0.03$), but was not replicated by recombinant GDF15.

Conclusions: Elevated circulating GDF15 is associated with poor prognosis in men with mCRPC receiving docetaxel and may be a marker of changes in the innate immune system in response to docetaxel resistance. These findings provide a strong rationale to consider GDF15 as a biomarker to guide a therapeutic trial of drugs targeting the innate immune system in combination with docetaxel in mCRPC.

KEYWORDS

biomarker, docetaxel, growth differentiation factor 15, metastatic castration-resistant prostate cancer, prognosis, therapeutic response

1 | INTRODUCTION

Over 350,000 men worldwide die from prostate cancer (PC) annually.[1] While androgen deprivation therapy is initially effective for metastatic disease, ultimately the disease progresses to the castration-resistant state. Docetaxel was the first agent to provide a survival benefit in metastatic, castration-resistant PC (mCRPC)[2,3] and, despite the addition of new therapeutic agents, remains central to the treatment paradigm. However, docetaxel significantly benefits only half of men, with PSA response rates of 45%–60%.[3–5] In an effort to improve this, multiple agents have been added to docetaxel over the past decade without success.[4,6–8] There is a need to identify biologically driven drug combinations to overcome docetaxel resistance.

There is increasing evidence that docetaxel resistance in mCRPC is in part mediated by tumor-associated macrophages (TAM) driving a TH2 anti-inflammatory response. Docetaxel resistance both in vitro and in patients is associated with an inflammatory response involving cytokines linked to macrophage recruitment and activation.[9] We identified a circulating cytokine signature (elevated growth differentiation factor [GDF]15, interleukin 4 [IL4], and IL6 at Day 21 after docetaxel), which was associated with poor response to docetaxel.[9] More recent data demonstrates that targeting TAMs through the colony-stimulating factor 1 (CSF-1) receptor antagonist PLX 2297 (pexidartinib) can overcome resistance to docetaxel in vitro and in vivo[10,11] by preventing recruitment and differentiation of macrophages into the M2 phenotype. Pexidartinib is now FDA-approved as first-line treatment for advanced tenosynovial giant cell tumors.[12] While this is a feasible treatment option, it would be optimal to know up-front which patients are most likely to benefit from this treatment.

Studies of circulating prognostic and predictive biomarkers in mCRPC have been small and variable. This study aimed to validate our previous findings[9] that circulating GDF15, IL4, and IL6 levels were associated with treatment response and prognosis in an internal independent validation cohort. These findings were then validated in a post hoc analysis of an external phase 3 clinical trial cohort.

2 | MATERIALS AND METHODS

2.1 | Study population

All participants provided written informed consent.

The internal validation (IntVal) cohort included prospectively enrolled patients with mCRPC receiving docetaxel as standard of care across seven sites in NSW, Australia (Figure S1). The study was approved by the Royal Prince Alfred Hospital Human Research Ethics Committee (HREC X19-0320) and was performed in accordance with the Declaration of Helsinki.

The external validation (ExtVal) cohort included a subset of patients from the SYNERGY study, a randomized, open label, multinational, phase 3 trial across 134 study centers in 12 countries.[4] Eligibility criteria, randomization, and study procedures have been previously reported.[4] Men with treatment-naive mCRPC were randomly assigned (1:1) centrally to receive docetaxel alone ($n = 512$) or with custirsen ($n = 510$), an antisense oligonucleotide inhibitor of clusterin production.[13] For this study, a subset of men (430/1022) were randomly selected from the study population and stratified according to whether they were in the control or the treatment arm of the study (all treated with docetaxel), opiate use for PC-related pain, and evidence of radiographic progression.[4]

2.2 | Sample collection

For the IntVal cohort, blood samples were collected according to a standardized operating protocol using BD Vacutainer tubes containing K_2EDTA for plasma separation at baseline and precycle 2 (Day 21).[14] For the ExtVal cohort, serum samples (baseline and/or precycle 3 [Day 42])

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were available for analysis. All serum/plasma samples were stored at −80°C and for this study were thawed for the cytokine assays.

2.3 | Cytokine assays

GDF15 (pg/mL) was measured using a previously described sensitive immunoassay[15] while IL4 and IL6 were measured by ELISA assays according to the manufacturer's instructions (R&D systems).

2.4 | Immune cell isolation and culture

Collection of healthy venous blood was approved by the Concord Hospital research ethics committee (HREC/11/CRGH/61). CD14+ monocytes were isolated from peripheral blood mononuclear cells (PBMC) as described previously.[16] Myeloid DC were isolated using EasySep™ Human Myeloid DC Enrichment Kit (Stemcell Technologies).

Isolated mDC and monocytes were incubated with 10% patient plasma in RPMI 1640 50 U/mL penicillin, 50 µg/mL streptomycin and 20 mM L-glutamine (Thermo Fisher Scientific) ± 100 ng/mL lipopolysaccharide (LPS) from *Escherichia coli K12* (LPS), (Invivogen) or 10% human pooled AB serum (Thermo Fisher) in RPMI ± GDF15 (R&D System) ± LPS 100 ng/mL.

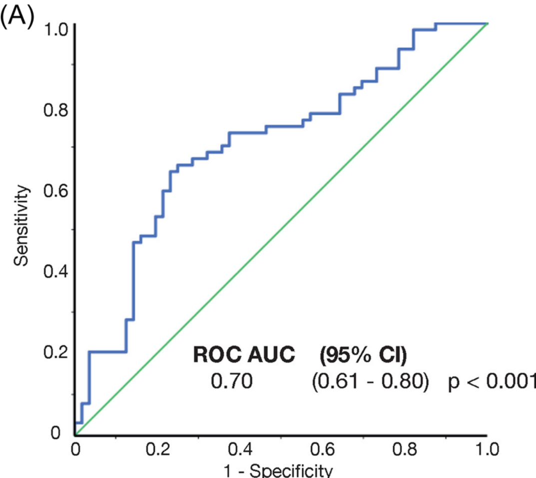
2.5 | Flow cytometry analysis

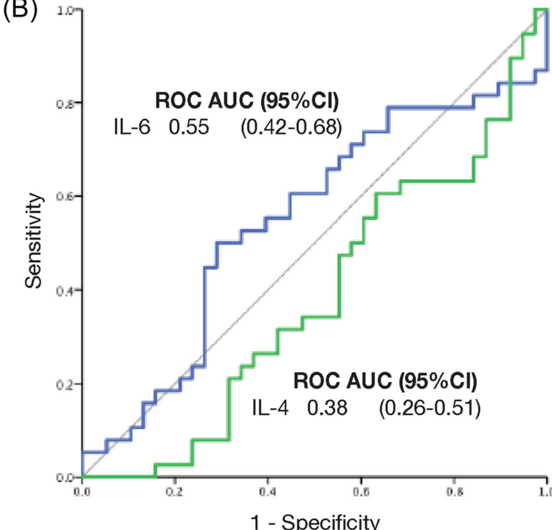
Cells were stained with the following antibodies: from BD Bioscience anti-human Lineage Cocktail 2, CD80 (L307.4), CD275 (MIH1), CD273 (MIH18), CD279 (EH12.1), from Biolegend CD86 (It2.2), HLA-DR (L243), CD366 (F38-2E2) and from Beckman Coulter CD83 (HB15a). Cell viability was determined using 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) added immediately before acquisition of flow cytometry data. Flow cytometry data was acquired on LSR Fortessa cytometer (BD Biosciences) and analyzed using FlowJo V10.5.0 (Treestar).

2.6 | Statistical analysis

Time to prostate-specific antigen (PSA) progression and overall survival (OS) were calculated from the date of commencement of docetaxel (Cycle 1 Day 1). PSA progression was defined as a PSA rise of 25% or more above the nadir or baseline value (if no fall from baseline is observed) with an absolute increase of at least 2 ng/mL, confirmed by a 2nd value three or more weeks later. If no fall in PSA was recorded, PSA progression was measured at least 12 weeks after commencing treatment. PSA response was defined as a fall in PSA of at least 50% at 12 weeks from commencing docetaxel (PSA50).[17]

Survival analyses were performed using the Kaplan–Meier and log-rank methods. For these analyses, GDF15 levels were dichotomized using the cut-point established in our prior exploratory study (5591 pg/mL).[9] The associations between GDF15 levels, OS and time





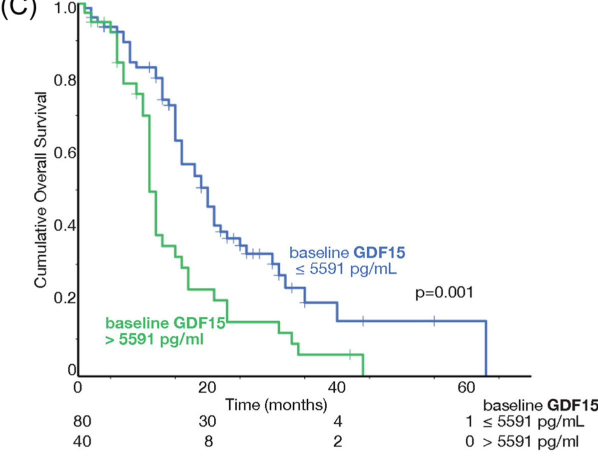


FIGURE 1 Internal validation cohort assessing the association between circulating growth differentiation factor (GDF)15/interleukin 4 (IL4)/IL6 and response to docetaxel chemotherapy. ROC curve for the ability of changes in (A) GDF15 and (B) IL4/IL6 at Day 21 to predict response to docetaxel (PD + SD vs. PR). Kaplan–Meier analysis demonstrating that (C) higher baseline GDF15 levels are associated with poorer overall survival. CI: confidence interval. [Color figure can be viewed at wileyonlinelibrary.com]

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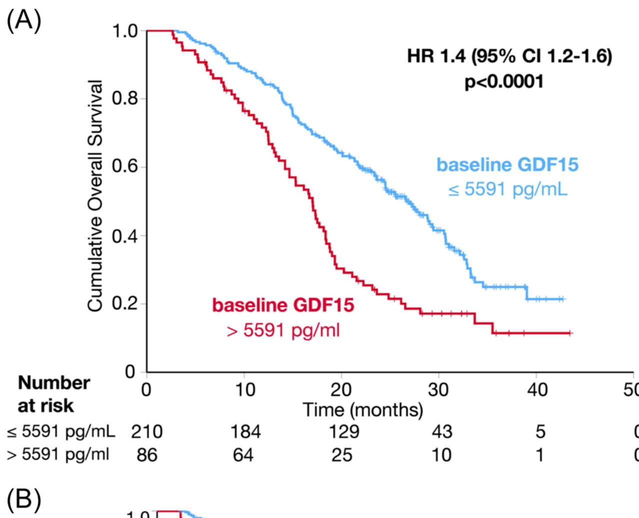
TABLE 1 Multivariable analysis for overall survival according to baseline GDF15 including established prognostic variables in internal (IntVal) and external (ExtVal) cohorts.

Variable	IntVal cohort hazard ratio (HR) (95% confidence interval [CI])	*p*-Value	ExtVal cohort HR (95% CI)	*p*-Value
GDF15[a]	1.3 (1.0–1.6)	0.02	1.4 (1.2–1.6)	<0.0001
Hemoglobin < 90 g/L	2.0 (0.6–6.6)	0.3	3.3 (1.6–7.0)	0.001
LDH > ULN	NA	NA	1.8 (1.4–2.5)	<0.0001
ALP > ULN	1.8 (1.1–3.0)	0.01	NA	NA
Karnofsky PS < 90	NA	NA	1.8 (1.3–2.4)	0.0002
Visceral metastases (present vs. absent)	1.1 (0.7–1.8)	0.6	1.8 (1.1–3.0)	0.02
Baseline PSA (ng/mL)[a]	0.9 (0.7–1.0)	0.1	0.97 (0.9–1.1)	0.5

Abbreviations: ALP, alkaline phosphatase; GDF15, growth differentiation factor 15; LDH, lactate dehydrogenase; PS, performance status; PSA, prostate-specific antigen.

[a]Continuous variable, log normalized.



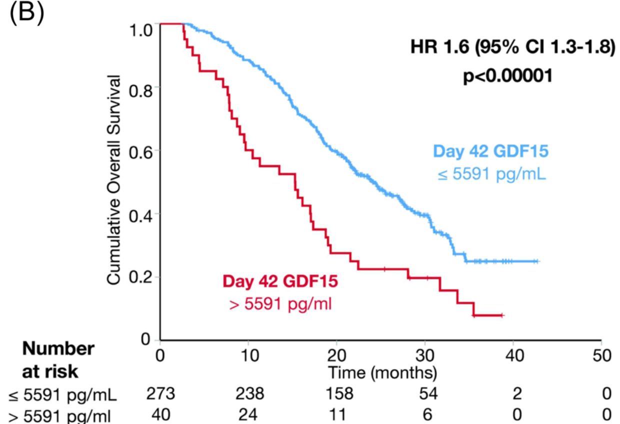


FIGURE 2 External validation cohort assessing the association between circulating growth differentiation factor (GDF)15 and response to docetaxel. Kaplan–Meier analysis demonstrating that (A) higher baseline GDF15 levels and (B) higher C3D1 GDF15 levels are associated with poorer overall survival. CI, confidence interval; HR, hazard ratio. [Color figure can be viewed at wileyonlinelibrary.com]

to PSA progression were analyzed by Cox regression. GDF15 levels were transformed to logarithm scale and assessed as a continuous variable. Correlations between GDF15 levels and PSA50 responses were analyzed by Mann–Whitney-*U* test and Pearson χ^2 test. Statistical analyses were performed using SPSS version 24 (IBM).

For in vitro experiments, statistical analyses were performed using Prism version 6 (GraphPad Software Inc.) using Mann–Whitney *U*-test or paired *t*-test as appropriate.

3 | RESULTS

3.1 | Patient characteristics

IntVal cohort: Men ($n = 121$) were prospectively recruited between December 2007 and July 2015. Paired plasma samples taken at baseline and Day 21 after commencing docetaxel were analyzed for GDF15, IL4, and IL6 by ELISA assay. After a median follow-up of 14 months (range 1–69 months) there were 88 (73%) deaths with a median OS of 16 months. The baseline clinical characteristics are detailed in Table S1.

ExtVal cohort: Serum samples from 430 men on the SYNERGY study at baseline and/or Day 42 after commencing treatment were analyzed for GDF15. Paired serum samples from both time points were available for 179 participants with 117 men having baseline only samples and 134 men having Day 42 samples only (Figure S1). Of the 430 patients included in the analysis, 226 (53%) were allocated to the study arm and 204 (47%) were allocated the standard therapy arm. As there was no OS benefit for the addition of custirsen to docetaxel[4] subsequent analyses were pooled across both arms.

In this patient subset, median follow-up for surviving patients was 27 months. At the time of primary analysis of the SYNERGY study, there were 272 (63%) deaths with a median OS of 23 months. The baseline clinical characteristics of our patient subset were similar to those in the overall SYNERGY study cohort (Table S1).[4]

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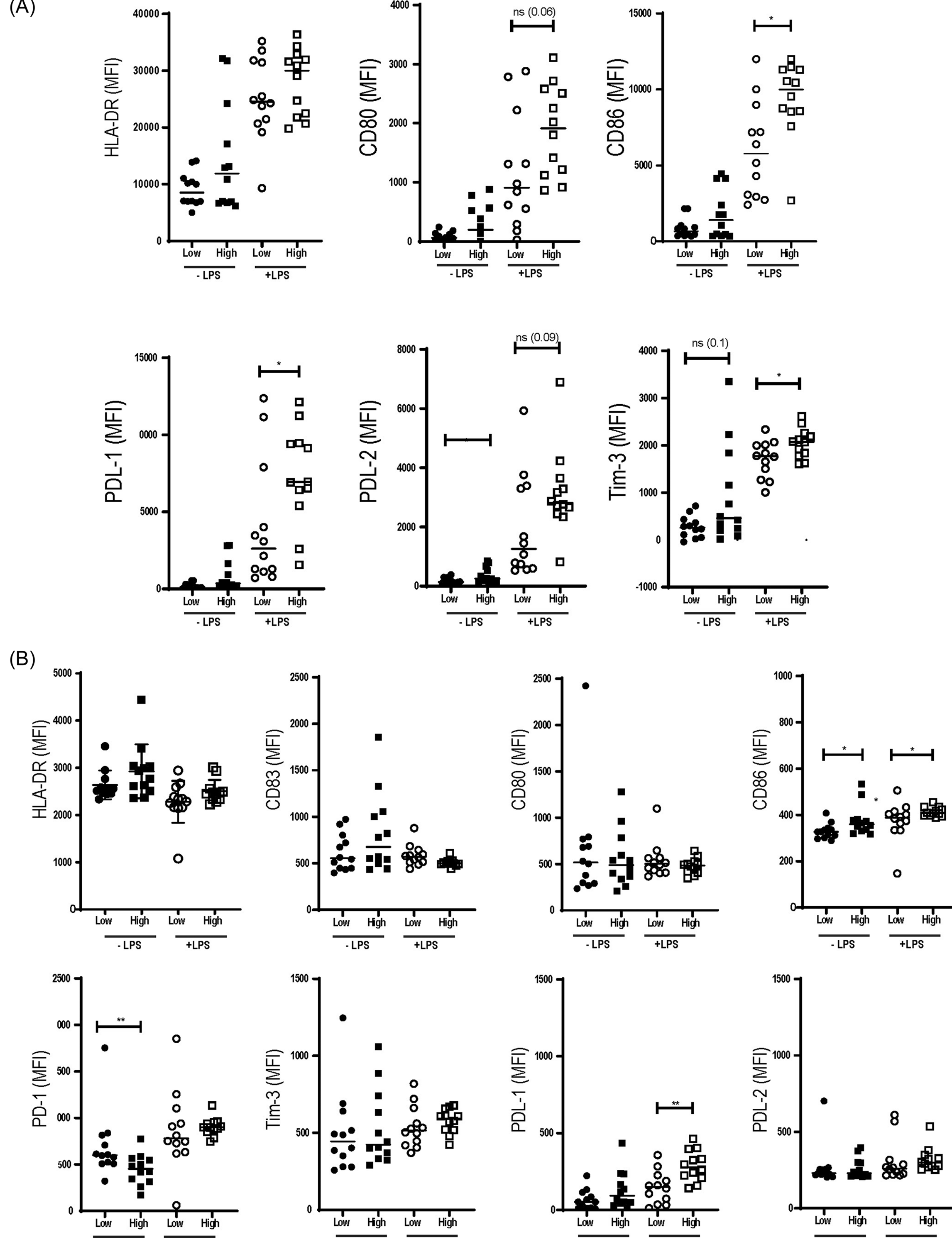

FIGURE 3 (See caption on next page).

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3.2 | GDF15/IL4/IL6 association with docetaxel response and outcomes in the internal validation cohort

After 1 cycle of docetaxel (IntVal cohort), nonresponders had significantly greater increases in circulating GDF15 ($p < 0.001$). On ROC analysis, chemoresistance was best predicted by early changes in GDF15 (ROC AUC: 0.70, 95% confidence interval [CI]: 0.61–0.80; $p < 0.001$, Figure 1A). In contrast to our previous analysis, IL4 and IL6 were not associated with response (Figure 1B). Furthermore, combinations of cytokine changes did not improve on GDF15 alone as an early biomarker of response.

Higher baseline GDF15 levels were associated with a shorter OS (HR: 1.2, 95% CI: 1.0–1.4; $p = 0.04$) (Figure 1C). While normal GDF15 levels are below 1150 pg/mL,[15,18] too few patients (IntVal $n = 12$, ExtVal $n = 44$) in our cohorts had normal levels to use this as a discriminator. The cut-point established in our prior exploratory study,[9] 5591 pg/mL, was used for Kaplan–Meier analysis. On multivariable analysis including established prognostic factors, higher baseline GDF15, as a continuous variable, was independently associated with shorter OS (Table 1; $p = 0.02$).

Most participants had a fall in their GDF15 levels after docetaxel. However, higher GDF15 levels after chemotherapy were associated with a shorter OS (HR: 1.3, 95% CI: 1.1–1.5; $p = 0.004$). On the basis of these results, only circulating GDF15 expression was evaluated in the ExtVal cohort.

3.3 | GDF15 association with docetaxel response and outcomes in ExtVal cohort

Higher baseline circulating GDF15 levels before docetaxel were again associated with shorter OS (HR: 1.4, 95% CI: 1.2–1.6; $p < 0.0001$) (Figure 2A). However, a baseline level ≤5591 pg/mL GDF15 did not predict for a PSA50 ($p = 0.7$). Circulating GDF15 levels remained an independent prognostic factor ($p < 0.0001$) when modeled with serum hemoglobin ($p = 0.001$), serum LDH ($p < 0.0001$), Karnofsky performance score ($p = 0.0002$), presence of visceral disease ($p = 0.02$) and baseline PSA ($p = 0.5$) (Table 1).

The second timepoint for GDF15 assessment was at Day 42, which is 3 weeks later than the IntVal cohort. Compared to baseline, lower GDF15 levels after chemotherapy (Day 42) were significantly associated with PSA50 response ($p = 0.002$). An increase in GDF15 after docetaxel correlated with a lack of PSA50 response ($p = 0.01$). Furthermore, GDF15 levels >5591 pg/mL at Day 42 were associated with shorter time to PSA progression (HR: 1.2, 95% CI: 1.0–1.4; $p = 0.02$). and shorter OS (HR: 1.6, 95% CI: 1.3–1.8; $p < 0.00001$, Figure 2B).

3.4 | Effect of high GDF15 on immune cells

As elevated circulating cytokines are associated with a pro-tumor environment, we assessed the effect of plasma from men in the IntVal cohort on myeloid dendritic cells (DCs) and monocytes. Cohorts from men with[1] high plasma GDF15 ($n = 12$) and[2] low plasma GDF15 ($n = 12$) were identified.

The addition of high GDF15 plasma, but not low GDF15 plasma to DC cultures resulted in a trend ($p > 0.1$) toward an increase in HLA-DR, CD80, CD86, PDL-1 and Tim-3 expression. PD-L2 surface expression was significantly increased on DC treated with high GDF15 plasma ($p = 0.03$) (Figure 4A). In DC cultures supplemented with LPS and high GDF15 plasma, but not low GDF15 plasma, significantly increased HLA-DR, CD86, PDL-1, and Tim-3 ($p = 0.01$, $p = 0.04$, $p = 0.03$ respectively, Figure 3A).

In monocyte cultures supplemented with low GDF15 plasma or high GDF15 plasma, there was no significant difference in HLA-DR, CD80, CD83, PDL-1, PDL-2, and Tim-3 expression ($p > 0.1$, Figure 3B). CD86 surface expression was significantly increased in monocytes treated with high GDF15 cultures compared to low GDF15 ($p = 0.01$). PD-1 surface expression was significantly reduced on monocytes treated with high GDF15 ($p = 0.01$) (Figure 3A). In monocyte cultures supplemented with LPS and high GDF15 plasma, but not low GDF15 plasma, CD86 and PDL-1 were significantly increased ($p = 0.04$, $p = 0.005$ respectively).

Next, we assessed if the effect of recombinant GDF15 alone could have the same effects on DCs and monocytes. The highest GDF15 plasma value was 214,773 pg/mL and was used in the assay at 10% concentration i.e. 21,477 pg/mL. GDF15 was titrated using the same conditions as the prior experiment. At 100 ng/mL, there was no consistent changes in either DC or monocytes demonstrating that GDF15 alone was not responsible for the phenotype (Figure 4).

4 | DISCUSSION

This study of circulating cytokines in two independent cohorts of men receiving docetaxel for mCRPC confirms that elevated levels of circulating GDF15 are associated with poor response to docetaxel and shorter OS. While IL4 and IL6 clearly have some part to play, their role is less closely linked to chemoresistance in human cohorts. In vitro experiments demonstrate that plasma from men with high GDF15 levels led to

FIGURE 3 Effect of plasma from growth differentiation factor (GDF)15 high versus GDF15 low patients on (A) myeloid dendritic cell (DC) and (B) monocyte cell surface phenotype. One hundred 1000 myeloid DC or monocytes isolated from a single healthy donor's peripheral blood mononuclear cells (PBMC) were incubated overnight with 10% plasma from each of 12 patients with GDF15 high expression (>5591 pg/mL) and 12 patients with GDF15 low expression (≤5591 pg/mL) with or without lipopolysaccharide (LPS). Analysis of surface markers expression was done using flow cytometry. Each dot represents the delta mean fluorescence index (MFI, MRI of marker-MFI of isotype control) from one analysis sample. Statistics done using Mann–Whitney *U* test. (Dots represent one sample, $^{*}p \le 0.05$, $^{**}p \le 0.01$).

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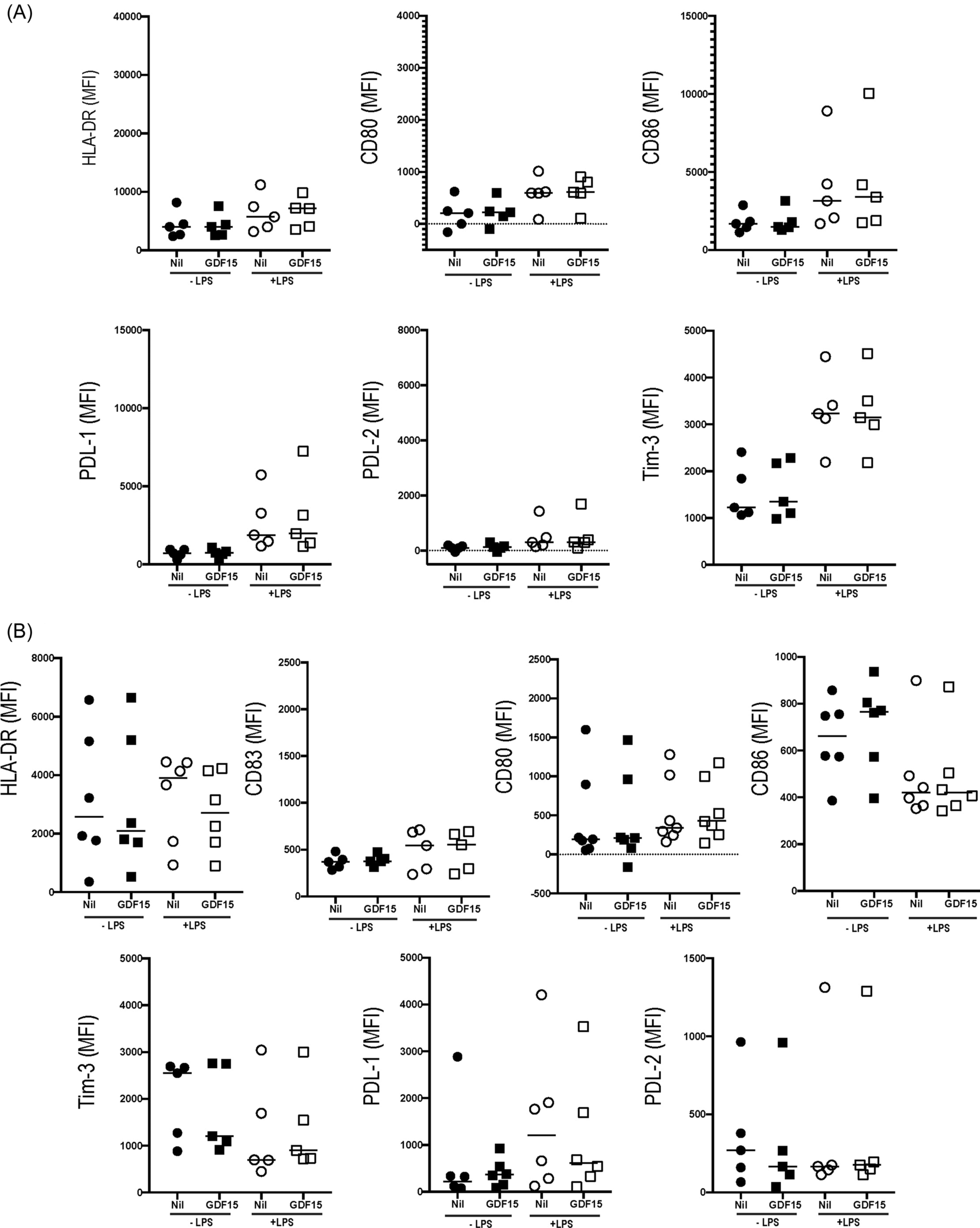

FIGURE 4 Effect of growth differentiation factor (GDF)15 on (A) myeloid dendritic cell (DC) and (B) monocyte phenotype. One hundred 1000 myeloid DCs or monocytes isolated from health donor peripheral blood mononuclear cells (PBMC) were incubated overnight with or without GDF15 100 ng/mL and with or without 100 ng lipopolysaccharide (LPS). Analysis of surface markers expression was done using flow cytometry. Each dot represents the delta mean fluorescence index from one healthy donor sample. Statistics done using paired *T*-test (*$p \le 0.05$, **$p \le 0.01$).

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increased CD86 on monocytes suggesting that activation of the innate immune system is associated with docetaxel resistance. Recombinant GDF15 alone could not re-capitulate this phenotype consistent with GDF15 being a marker of a wider immune response in these men.

The link between GDF15 and docetaxel resistance was identified more than a decade ago. In vitro analyses have shown that in androgen-independent prostate cancer (AIPC) cell lines, PC3 and DU145, forced overexpression of GDF15 or treatment with recombinant GDF15 confers docetaxel resistance.[19–21] Knockdown of GDF15 by siRNA in docetaxel-resistant cell lines (PC3-Rx) restores docetaxel sensitivity.[19] Co-cultures of docetaxel-resistant cell lines (PC3-Rx) with monocytes induces secretion of GDF15 which is further enhanced by docetaxel treatment.[9] When cocultured with osteocytes, AIPC cell lines induce osteocytes to secrete GDF15, in turn promoting proliferation, invasion, and migration by AIPC cells via early growth response 1 (EGR1) receptor signaling.[22] In men with mCRPC, we have now confirmed that higher circulating GDF15 levels of >5591 pg/mL at baseline or Day 42 after starting docetaxel are associated with poorer overall survival compared to lower levels.

There is abundant evidence across almost all cancer subtypes that high GDF15 levels confer a poor prognosis.[23] In other cancers, GDF15 has also been implicated in resistance to carboplatin.[24] However, there appears to be a differential role in early prostate carcinogenesis compared to late PC progression. GDF15 enhances antitumor immunity, which can prevent the development of cancer.[25] On the other hand, GDF15 appears to have an integral role in aiding cancers to evade immune surveillance in the more advanced setting including repolarising macrophages to an anti-inflammatory, tumor promoting M2 phenotype.[26]

Our in vitro *data* suggests that GDF15 is a marker of the overall circulating immune response modulating resistance to docetaxel. Plasma from men with high GDF15 expression induced significant expression of the activation marker CD86 on monocytes and some expression of CD83, CD80, and CD86 with significant increase in PDL-2 on DCs. In the presence of LPS, high GDF15 plasma further activated both DC and monocytes with higher CD86 expression. The net result of these data is that men with high GDF15 and docetaxel resistance may have upregulation of the innate immune system.

Targeting docetaxel resistance remains one of the conundrums of the mCRPC treatment paradigm. There have been many unsuccessful phase 3 studies targeting pathways such as apoptosis[4,27] and angiogenesis,[6,28] however, none have had a companion biomarker. Based on our data, we now have level 2 evidence[29] for circulating GDF15 as a marker of poor prognosis after treatment with docetaxel for mCRPC. This may be due to an upregulated innate immune response in these men that promotes resistance to docetaxel as seen in preclinical data.[10,11] The next step is to target the innate immune system in men with mCRPC and elevated GDF15 level after two cycles of docetaxel by adding a drug such as the CSF-1 receptor antagonist, PLX 2297 (pexidartinib). This strategy aims to maximize the potential of this combination by choosing a group of men who are most likely to benefit.

5 | CONCLUSIONS

This is the first study to demonstrate level 2 evidence that circulating GDF15 is a prognostic biomarker in docetaxel-treated mCRPC. GDF15 is likely to be a marker of changes in the immune system in response to docetaxel resistance rather than a druggable target. These data provide clinical validation and the rationale to consider GDF15 as a biomarker to guide a therapeutic trial of drugs targeting the innate immune system in combination with docetaxel.

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CONFLICT OF INTEREST STATEMENT

Authors Samuel N. Breit and David A. Brown are inventors on patents related to MIC1/GDF15 owned by St. Vincent's Hospital Sydney. These patents have been commercialized. The other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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