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Androgen deprivation therapy promotes an obesity-like microenvironment in periprostatic fat

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Abstract

Prostate cancer is a leading cause of morbidity and cancer-related death worldwide. Androgen deprivation therapy (ADT) is the cornerstone of management for advanced disease. The use of these therapies is associated with multiple side effects, including metabolic syndrome and truncal obesity. At the same time, obesity has been associated with both prostate cancer development and disease progression, linked to its effects on chronic inflammation at a tissue level. The connection between ADT, obesity, inflammation and prostate cancer progression is well established in clinical settings; however, an understanding of the changes in adipose tissue at the molecular level induced by castration therapies is missing. Here, we investigated the transcriptional changes in periprostatic fat tissue induced by profound ADT in a group of patients with high-risk tumours compared to a matching untreated cohort. We find that the deprivation of androgen is associated with a pro-inflammatory and obesity-like adipose tissue microenvironment. This study suggests that the beneficial effect of therapies based on androgen deprivation may be partially counteracted by metabolic and inflammatory side effects in the adipose tissue surrounding the prostate.

Key Words

- ▶ prostate
- ▶ cancer
- ▶ obesity
- ▶ adipose tissue
- ▶ transcriptomics
- ▶ transcriptomic econvolution

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Introduction

For over 80 years, androgen deprivation by surgical or medical castration has been the cornerstone of treatment for advanced prostate cancer (1). As new cytotoxic and androgen receptor-targeted therapies have been developed, demonstrating survival benefit in combination with androgen deprivation in a number of clinical settings, the duration a patient can expect to be in a castrated state

prior to death has been extended significantly (2). Given that androgen signalling is important for homeostasis in a number of different organ systems, it is not surprising that both short- and long-term use is associated with a number of deleterious effects (3).

Forefront of these is the association of androgen deprivation with metabolic syndromes such as diabetes

mellitus (4) and obesity (5), as androgens play a key role in the regulation of intermediate metabolism and tissue composition (6). Increased fat tissue mass (known in conjunction with loss of muscle mass as sarcopenic obesity) is one of the main metabolic side effects of androgen deprivation therapy (ADT) (7), even for short-term treatment (8, 9, 10). At the molecular level, lack of androgen-related hormones leads to changes in tissue lipid composition and decreased insulin sensitivity (4). For example, gonadotropin-releasing hormone agonists have been shown to alter tissue lipid profiles with cholesterol levels, triglycerides and high-density lipoproteins shown to increase up to 10.6, 25 and 8–20%, respectively (8, 10).

The promotion of an obese-like phenotype by androgen deprivation is highly clinically relevant, as obesity (expressed as BMI) is itself associated with the development of prostate cancer, post-prostatectomy biochemical failure and risk of death from prostate cancer. Although the link between elevated BMI and increased risk of prostate cancer is still controversial (11, 12, 13), several studies have found a positive association between BMI and cancer grade and/or stage at the time of radical prostatectomy (14, 15, 16). Two recent studies identified an association between BMI and biochemical failure rates following radical prostatectomy, based on a large-scale, multi-ethnic cohort (13, 17). The relationship between BMI and prostate cancer-specific mortality is also widely supported (18, 19, 20, 21).

Although the connection between ADT, obesity and prostate cancer progression is well established in clinical settings, a molecular understanding of the changes in adipose tissue associated with castrating therapies is still missing, in part due to a paucity of appropriate clinical specimens. This is especially important for periprostatic adipose tissue due to its proximity to the cancer site and its potential to influence prostate hormonal and immune homeostasis (22). Here for the first time, based on a unique cohort of patients with 6-month profound androgen suppression and receptor blockade, we performed an integrative study of the molecular and cellular changes in periprostatic fat associated with androgen deprivation. In this study, we show that ADT is associated with a pro-inflammatory and obesity-like adipose tissue microenvironment.

Materials and methods

Ethics statement

The collection and use of tissue for this study had Epworth Healthcare institutional review board approval

and patients provided written informed consent (HREC approval number 34506).

Study cohort selection

ADT-treated patients ($n=11$) were recruited from an open-label neoadjuvant phase II study in which patients with high-risk disease received a ‘supercastration’ regimen consisting of degarelix 240/80 mg subcutaneously every 4 weeks; abiraterone acetate 500 mg orally daily titrating upwards every 2 weeks by 250 mg to a final dose of 1000 mg daily; bicalutamide 50 mg orally daily and prednisolone 5 mg orally twice daily for a total of 6 months (Australian New Zealand Clinical Trials Registry 12612000772842). Untreated patients with similar pre-treatment characteristics were obtained from a prospective prostatectomy biorepository (22, 23). Prior to ligation of the dorsal venous complex and prostate pedicles, the anterior prostate was defatted and the specimen was removed immediately, placed in a sterile container and transferred on ice for long-term storage in the vapour phase of liquid nitrogen. Patients were risk categorised using the CAPRA scoring system, which uses pre-treatment clinical and pathological variables (including age, serum PSA level, biopsy tumour grade, clinical stage) to predict the risk of bone metastasis and prostate cancer-specific mortality (24). Differences between patient groups were assessed by the Mann–Whitney or chi-squared test as appropriate.

Gene expression screen

A total of 50–100 μ g of adipose tissue was separated from fresh frozen samples stored at -160°C . RNA was isolated using the Qiagen RNeasy Lipid Tissue Mini Kit and eluted in 35 μ L nuclease-free water. 0.5–1 μ g of total RNA was used as the input for cDNA library synthesis using TruSeq RNA Sample Prep Kit v2 (Illumina), and libraries were constructed according to manufacturer’s instructions. Samples were sequenced on a HiSeq 2500 (Illumina) using 101 base paired-end chemistry, aiming for 50 million mapped paired-end reads per sample.

Data pre-processing and mapping

The RNA-sequencing quality for each sample was controlled using the FastQC algorithm (25). Reads were trimmed for Illumina adapters and low-quality fragments using the Trimmomatic algorithm, and short reads filtered out from the pools according to default settings (26).

The remaining reads were aligned to the reference genome (hg19) with the STAR aligner using default settings (27). The gene abundance for each sample was quantified in terms of reads per gene (read-count) using featureCounts (28). Low abundance genes were filtered from the analysis, if not present in at least 0.5 parts per million in two-thirds of the samples in each treatment group (i.e., treated and naïve).

Differential expression and gene set enrichment analyses

Considering the sparse batch distribution, the gene abundances were adjusted for unknown variation using RUVseq with one unwanted covariate (using default settings) (29). The resulting covariate matrix for the unwanted covariate was appended to the design matrix (i.e. treated vs naïve, plus the intercept term); then, all samples were tested for differential transcription using the edgeR package (30), considering differentially transcribed genes with a false discovery rate <0.05. Ensemble pathway analyses were performed using the algorithm EGSEA (31). In order to test for the enrichment of an obesity molecular phenotype among the differentially transcribed genes, an *ad hoc* signature data set (46) was queried using the algorithm GSEA (32).

Differential tissue composition analyses

The associations between (i) the abundance of stromal and immune cell types within the tissue and (ii) the treatment status (i.e., treated or naïve) was inferred using

two distinct approaches. Both approaches included a two-step inference, where the cellular composition of each sample is inferred first (i.e., the proportion of several cell types within the tissue sample), and an association analysis is performed integrating such inference with the treatment status. The first approach applied the algorithm Cibersort (33), for the inference of tissue composition, in combination with DirichletReg (34), for the regression of the proportional estimates produced by Cibersort. Considering that Cibersort was designed mainly for microarray data, and only for PBMC cell types, a custom probabilistic Bayesian model was also implemented (Fig. 1) based on the Markov chain Monte Carlo probabilistic framework Stan (35), which natively models RNA sequencing data and performs association analysis in an integrative manner preserving uncertainty information between the two steps. This probabilistic model can be described by a joint probability density formula and a series of sampling statements (Supplementary Figure 1, see section on [supplementary data](#) given at the end of this article).

qRT-PCR validation

In order to validate the methodology used for the inference of differential transcription, qRT-PCR was used for an independent observation of gene transcript abundance. A total of nine differentially transcribed genes were selected for validation with qRT-PCR, based on false discovery rate (<0.05), log fold change (>2) and on the absence of clear outliers. The qRT-PCR validation was performed using 1 µL of cDNA, 0.5 µL qRT-PCR primers (see below), 5 µL of

$$(1) P(\sigma)P(\phi)P(\delta)\prod_{r=1}^R\prod_{p=1}^P P(\alpha_{r,p}|\delta)\prod_{p=1}^P\prod_{s=1}^S P(\pi_{p,s}|X_s,\alpha_p,\phi)\prod_{s=1}^S\prod_{g=1}^G P(Y_{s,g}|x_g,\pi_s,\sigma_s,\theta^*)$$

$$(1) P(Y|x,\pi,\sigma,\theta^*) \sim \text{lognormal}(x * \pi, \sigma)$$

$$(2) P(\pi|X,\alpha,\phi) \sim \text{Dirichlet}(\hat{Y})$$

$$(3) P(\alpha|\delta) \sim \text{Dirichlet}([\delta_1, \dots, \delta_k])$$

$$(4) P(\sigma) \sim \text{normal}(0, 0.1)$$

$$(5) P(\phi) \sim \text{normal}(1, \dots)$$

$$(6) P(\delta) \sim \text{cauchy}(1, 2)$$

$$(7) \hat{Y} = \text{softmax}(\hat{Y}) * \sigma$$

$$(8) \hat{Y} = X \cdot \alpha$$

$$(9) \text{softmax}(z)_j = \frac{e^{z_j}}{\sum_{k=1}^K e^{z_k}} \text{ for } j = 1, \dots, K$$

Figure 1

Probabilistic Bayesian inference model. The parameter α represents the rates of change of each cell type category along the biological conditions. The parameter π represents the matrix of proportions for each cell type category and sample. The parameters σ , ϕ and δ define the noise model. The point estimate and credible intervals for both cell type proportions and trends of change are calculated from the posterior distribution.

TaqMan Fast Advanced Master Mix (Applied Biosystems) and 3.5 µL of UltraPure distilled water (Gibco). The primers, including *ART3* (Hs00922621_m1), *CSDC2* (Hs00411093_m1), *DIO2* (Hs05050546_s1), *FCGR1B* (Hs00174081_m1), *LYZ* (Hs00426232_m1), *OR51E2* (Hs00258239_s1), *SLC16A12* (Hs01584854_m1), *SUSD5* (Hs01394532_m1), and *TRIM67* (Hs01595609_m1), were pre-designed and commercially available from Applied Biosystems. Samples were run on a 384-well plate using a Viia7 qRT-PCR machine (Applied Biosystems) under the following conditions: UNG incubation at 50°C for 2 min; polymerase activation at 95°C for 20 s; followed by 40 cycles of denaturation at 95°C for 1 s; anneal/extend at 60°C for 20 s. Expression levels of target genes were normalised to the geometric mean of *GAPDH* (Hs00266705_g1, Applied Biosystems), *TBP* (Hs00427621_m1, Applied Biosystems) and *POLR2A* (Hs00427621_m1, Applied Biosystems) using the formula $2^{-\Delta C(T)}$. One-sided Student's *t*-test was used for hypothesis testing; then, Bonferroni multiple-test correction was applied to the produced *P* values.

Results and discussion

Patient characteristics

The treated and naïve groups comprised 11 and 10 patients respectively; their clinical and pathological characteristics are shown in [Table 1](#). Given that pre-operative risk assessment is frequently inaccurate (36, 37), being biased towards underestimation of tumour grade and stage, patients in the high-risk cohort were selected based on the stage, grade and volume of tumour in the prostatectomy specimen. All patients in the treated cohort had high-risk disease at the time of initial assessment, although the ultimate response to androgen deprivation was highly variable. On average, patients exposed to androgen suppression experienced a 3.3% increase in body weight/BMI from baseline over the course of treatment.

Differentially transcribed genes represent three main functional groups

The RNA sequencing libraries had an average of 55 million reads across the 21 samples. All samples had a Phred quality score exceeding 28 following filtering and trimming (25). As expected, the distribution of the multidimensional scaling (MDS) analysis (38) including both treated and naïve groups showed the improvement in clustering obtained through the removal of unwanted

variation (RUVseq; [Fig. 2A](#) and [B](#)). However, the overall magnitude of differences between the two groups was low (i.e., log fold difference <3; [Fig. 2B](#) and [C](#)). No significant difference was found between the two treatment categories for BMI or CAPRA-S risk score distributions (adjusted *P* value=1.0 and 1.0 respectively; Supplementary Fig. 1).

A total of 70 genes were identified as differentially transcribed (false discovery rate <0.05; Supplementary Table 1), characterised by a median fold change of 3.23. Of these, 49 genes were characterised by a fold change greater than 2. Among the differentially transcribed genes with fold change greater than 2, three recurring biological processes (from grouping analogous gene ontology annotations; GO (39); Supplementary Table 2) were identified: hormonal and fat homeostasis (*n*=8), inflammation (*n*=8) and neural plasticity (*n*=4) ([Fig. 2D](#)). Several genes involved in cholesterol metabolism were

Table 1 Clinical characteristics of study cohort.

	Naïve	Treated	<i>P</i> value
Age (years)			
Median	66	65	0.79
Range	49–72	63–72	
PSA (ng/dL)			
Median	7.5	14.4	0.46
Range	2.7–27	4.4–95	
<10	7	5	0.35
10–20	2	2	
>20	1	4	
Clinical Stage			
cT1	3	2	0.08
cT2	7	4	
cT3	0	5	
Biopsy grade			
ISUP2	2	1	0.16
ISUP3	3	0	
ISUP4	2	3	
ISUP5	3	7	
Pathological stage			
pT0	0	1	0.13
pT2	0	3	
pT3	10	8	
Prostatectomy grade			
ND	0	1	0.26
ISUP1	0	2	
ISUP2	0	1	
ISUP3	3	1	
ISUP4	1	2	
ISUP5	6	5	
Tumour volume			
Median	7.1	1	0.012
Range	0.7–17.8	0–9.3	
BMI (kg/m ²)			
Mean	26.9	28.2	0.67
s.d.	2.9	4	

BMI, body mass index; PSA, prostate-specific antigen.

Enriched inflammatory signature

Overall, the gene enrichment analysis performed by EGSEA showed a pro-inflammatory signature for all query data sets (e.g. Hallmarks, Gene Ontology, KEGG and Immune Signatures; Table 2) (31). The pathways within the immune signature data set included *IL6/JAK/STAT3* signalling, interferon gamma response, positive regulation of immune response and antigen processing and presentation. Specifically for the immune signature dataset, transcriptional changes pointed to the differentiation of immature immune cell types (i.e., immature dendritic cells and monocytes), as well as neutrophil and mast cell activation.

Consistent with the gene enrichment analyses, the differential tissue composition analysis based on our Bayesian inference model showed a positive association between overall immune cell abundance and treatment status (Fig. 3A). In the two approaches employed for differential tissue composition analysis, monocyte-derived cells dominated the immune population within adipose tissue across the treated and naïve cohorts. Signatures of macrophages, monocytes and granulocytes were enriched by our model within the immune cell population in treated patients compared to naïve. This inference was partially consistent with that of the Cibersort-DirichletReg approach (i.e. for monocytes and macrophages; Fig. 3B).

The latter approach uniquely identified an association involving CD4 memory resting, NK cells resting and mast cells resting. Although a significant enrichment of CD8+ T-cells in treated patients was not observed using our statistical model and the Cibersort-DirichletReg approach, a positive association appears to exist when observing the distributions of the estimated cell type proportions (Supplementary Fig. 3). As expected, considering the absence of a robust adipocyte transcriptomic signature within the model, the fibroblast cell type appears to have captured the adipocyte transcriptomic profile (Supplementary Fig. 3). The differences observed in the average estimated proportions for immune cell types between Cibersort and our statistical method are in part due to the inclusion of non-immune cells (e.g. fibroblasts, endothelial and epithelial) in our model, while Cibersort models selectively estimate immune cells as composing the totality of the tissue.

Enriched obesity signature

The analysis of a previously published obesity transcriptional signature for adipose tissue (46) revealed a positive association with androgen deprivation treatment independent of BMI (false discovery rate of 8.4×10^{-3} ; Fig. 4). Within the ten top ranked genes

Table 2 EGSEA results.

GeneSet	Direction	P value	P adj
Hallmark signatures			
Hallmark allograft rejection	Up	$<1.0 \times 10^{-16}$	$<1.0 \times 10^{-16}$
Hallmark kras signalling up	Up	$<1.0 \times 10^{-16}$	1.0×10^{-06}
Hallmark inflammatory response	Up	$<1.0 \times 10^{-16}$	$<1.0 \times 10^{-16}$
Hallmark IL6 jak stat3 signalling	Up	8.0×10^{-06}	5.0×10^{-05}
Hallmark interferon gamma response	Up	$<1.0 \times 10^{-16}$	$<1.0 \times 10^{-16}$
Gene ontology			
GO regulation of innate immune response	Up	2.0×10^{-06}	3.8×10^{-05}
GO innate immune response	Up	$<1.0 \times 10^{-16}$	9.0×10^{-06}
GO positive regulation of defence response	Up	4.0×10^{-06}	8.4×10^{-05}
GO positive regulation of immune response	Up	$<1.0 \times 10^{-16}$	9.0×10^{-06}
GO immune system process	Up	4.9×10^{-05}	7.1×10^{-4}
KEGG			
hsa04612 Antigen processing and presentation	Up	$<1.0 \times 10^{-16}$	$<1.0 \times 10^{-16}$
hsa05152 Tuberculosis	Up	1.7×10^{-05}	1.6×10^{-4}
hsa05164 Influenza A	Up	2.2×10^{-05}	2.0×10^{-4}
hsa05332 Graft-versus-host disease	Up	$<1.0 \times 10^{-16}$	$<1.0 \times 10^{-16}$
hsa05140 Leishmaniasis	Up	$<1.0 \times 10^{-16}$	$<1.0 \times 10^{-16}$
Immune signatures			
GSE7509 Genes downregulated in immature dendritic cells	Up	$<1.0 \times 10^{-16}$	$<1.0 \times 10^{-16}$
GSE2706 Genes downregulated in comparison of unstimulated DC	Up	$<1.0 \times 10^{-16}$	$<1.0 \times 10^{-16}$
GSE19888 Genes upregulated in HMC-1 (mast leukaemia) cells	Up	$<1.0 \times 10^{-16}$	$<1.0 \times 10^{-16}$
GSE34156 Genes downregulated in monocytes	Up	$<1.0 \times 10^{-16}$	$<1.0 \times 10^{-16}$
GSE37416 Genes upregulated in activated neutrophils	Up	7.0×10^{-06}	9.7×10^{-05}

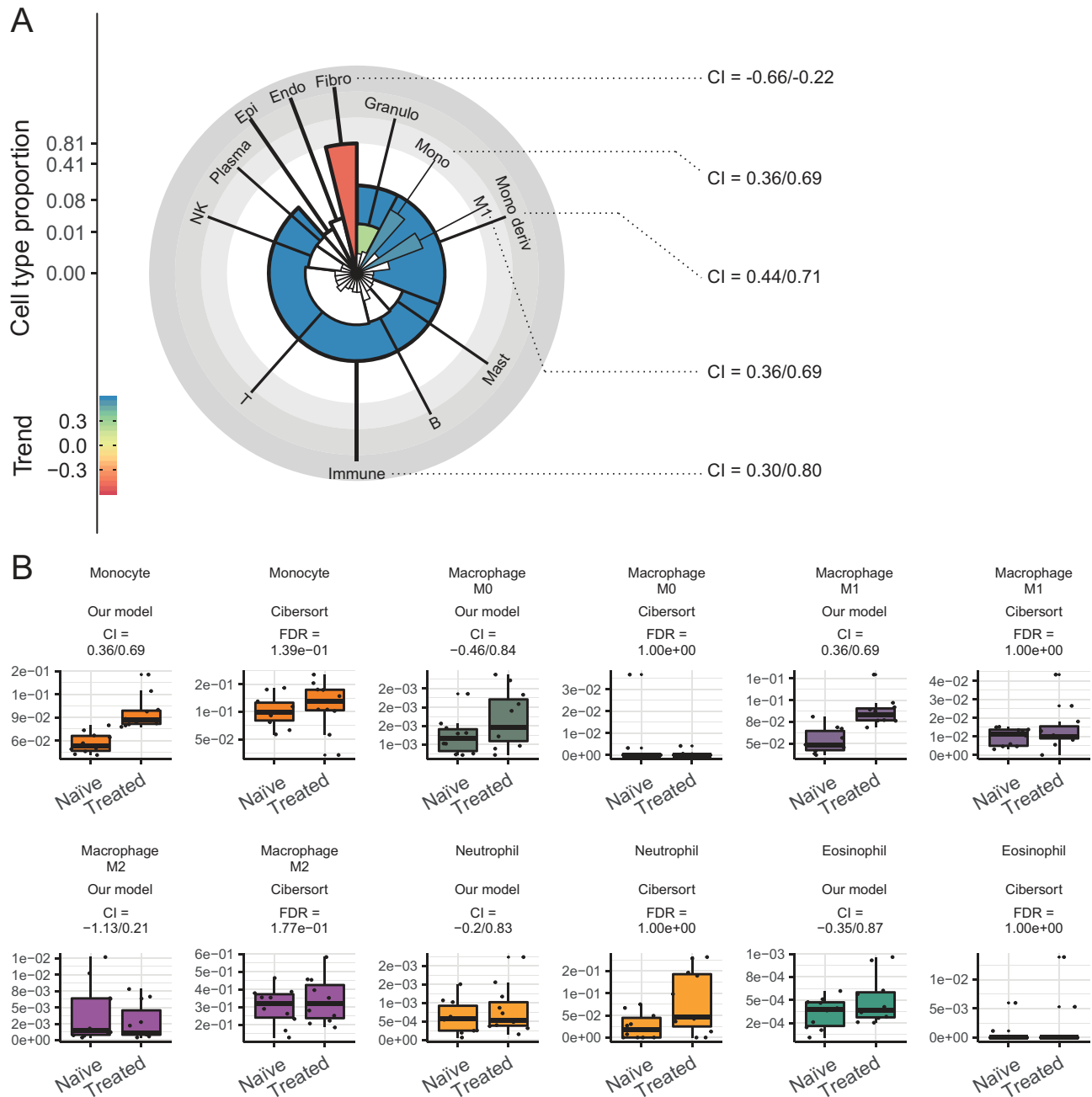


Figure 3

Differential tissue composition analysis. (A) Polar plot representing the overall cell type abundance (i.e. radius dimension) and the significant associations with androgen deprivation therapy (i.e. white for non-significant associations). Cell types are labelled if more abundant than 1%. CI = 95% credible interval of the association. (B) Boxplots of the inferred cell type proportions by our Bayesian probabilistic model and Cibersort, for the cell types that correspond or are part of significantly differentially abundant cell type categories (e.g. the differentially abundant category ‘granulocytes’ include eosinophils and neutrophils) between the two treatment categories (i.e., treated and naïve) according to our model. FDR = false discovery rate linked to an association being different non null.

present in the obesity signature, the majority were linked to inflammation (Supplementary Table 2), including Fc fragment of IgG-binding protein (*FCGBP*), lysozyme (*LYZ*), chemokine ligand motif 10 (*CXCL10*), myeloid cell nuclear differentiation antigen (*MNDA*), toll like receptor 8 (*TLR8*)

and a member of the STAT family (*STAT1*), which is activated by various ligands including interferon alpha, interferon gamma (*IFN γ*), epidermal growth factor (*EGF*), platelet derived growth factor (PDGF) and interleukin 6 (IL6). The third top ranked gene (included in the obesity signature)

is a key regulator of hormonal homeostasis (*DHRS9*), which is able to convert 3-alpha-tetrahydroprogesterone to dihydroxyprogesterone and 3-alpha-androstanediol to dihydroxyprogesterone in the cytoplasm (47); also, it is a marker for regulatory macrophages (48). Regulatory genes for calcium homeostasis were also present, including S100 calcium-binding protein A1 (*S100A1*) and stanniocalcin 2 (*STC2*), which regulate renal and intestinal calcium and phosphate transport (49).

Conclusions

The prostate gland is enveloped in adipose tissue, and over the last decade a number of lines of evidence suggest that paracrine interactions between this fat depot and prostate epithelium play a role in prostate cancer development and/or progression. For instance, tumour cell invasion into the periprostatic fat compartment where direct cell-to-cell interaction can occur has been reported to be a stronger determinant of cancer recurrence than acquisition of the ability to invade across tissue boundaries (50). Periprostatic adipose tissue has been shown to elaborate

a number of cytokines including IL6, osteopontin, and TNF-alpha, that promote prostate tumour cell migration and invasion (51, 52), and at least for IL6 correlates with downstream pathway activation in high-grade tumours (53). In addition, there is evidence of a positive feedback loop, with conditioned media from prostate cancer cells significantly increasing the secretion of these cytokines from adipose tissue explants (52).

The role of adipose tissue in prostate cancer progression is perhaps best understood in the context of obesity, where numerous clinical studies report positive associations between BMI and high-risk pathological findings at prostatectomy as well as adverse clinical outcomes post treatment (54). Obesity induces a persistent inflammatory and hormone-rich tissue microenvironment that contributes to high-risk disease (55, 56). ADT is a known cause of increased fat body mass (14, 15, 16); yet, the cellular and molecular processes that are altered in association with ADT, especially in the periprostatic adipose tissue microenvironment, have not been completely resolved. In this study, we showed that profound ADT is associated with a pro-inflammatory adipose tissue microenvironment, as well

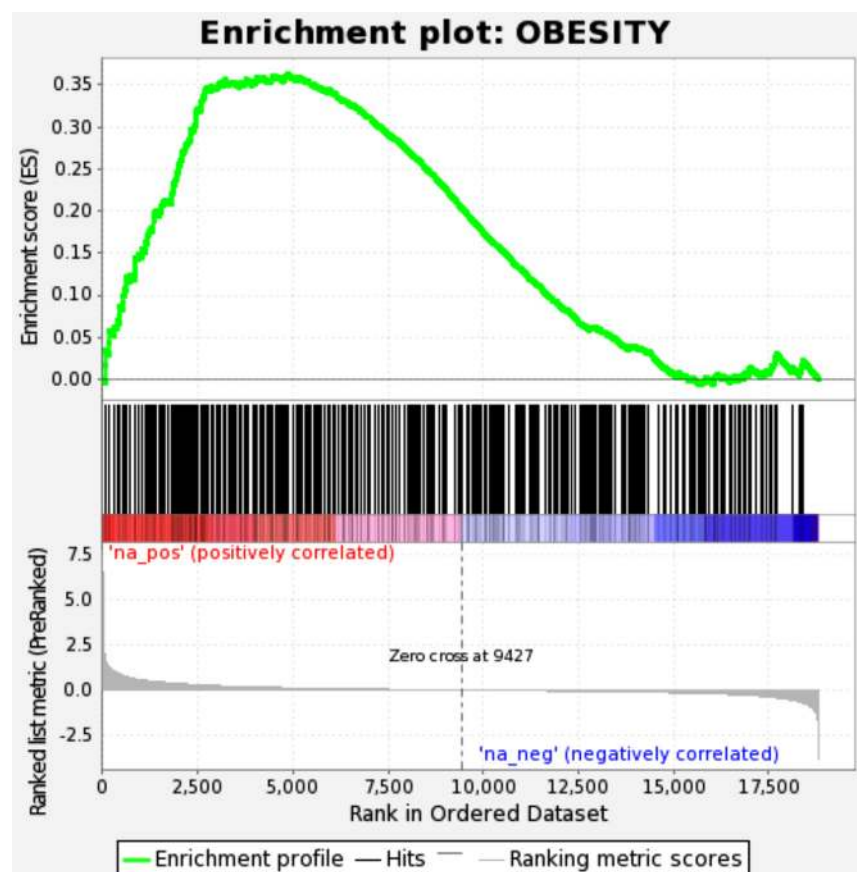


Figure 4
GSEA enrichment plot showing the significant enrichment of the obesity signature among the most differentially transcribed genes.

as with altered obesity-related gene transcription linked with cholesterol and hormonal homeostasis. Both differential tissue composition and gene enrichment analyses pointed to an enrichment of infiltrating immune cell types within the tissue as the predominant cause of this difference. Monocytes and macrophages had the greatest presence within the periprostatic adipose tissue, compared with other immune cells. The abundance of these immune cell types was positively associated with androgen deprivation, suggesting their infiltration of the tissue, which is consistent with *in vivo* studies (57). Macrophages have been shown to interact with adipose tissue in a paracrine manner, where TNF- α secretion from macrophages interferes with adipocyte insulin signalling and induces fatty acid lipolysis, which commences a vicious inflammatory cycle and contributes to insulin resistance (58). Furthermore, an elevated blood monocyte count is an independent prognostic predictor for poor prostate cancer outcome in cancer-specific and overall survival studies (59, 60). These findings are perhaps not surprising, given numerous reports describing the anti-inflammatory properties of androgen receptor signalling. How this is mediated is not clear, although testosterone has been reported to attenuate both Th2 and Th17 inflammatory responses (61, 62), as well as directly suppressing the secretion of monocyte chemoattractant protein-1 in adipocytes, a key cytokine that promotes monocyte infiltration (63).

There are a number of limitations to our study that merit enumeration, particularly the lack of orthogonal validation at the protein level for pathway and/or cell type tissue enrichment observed in our expression profiles. However, we note that previous studies have confirmed that alterations of inflammatory signalling identified expression data are accurately reflected by protein level changes in the abundance of key cytokines, including IL6 (64), and extensive validation studies have shown that the expression of key inflammatory cell markers are consistent with expression data from RNA-seq analysis (65) (www.proteinatlas.org). In addition, ideally we would use paired pre- and post-treatment samples from the same patients for analysis. However, this was not practical for clinical reasons, as collection of sufficient quantities of periprostatic adipose tissue for the type of exploratory analysis described is only possible at the time of prostatectomy. We have therefore tried to match patients as much as possible based on their pre-treatment clinical and pathological characteristics as described.

Taken together, our study demonstrates that androgen deprivation promotes an inflammatory and obesity-like

microenvironment in periprostatic fat and suggests that the beneficial effect of ADT may be partially counteracted by metabolic and inflammatory side effects in the adipose tissue encompassing the prostate. This may be particularly pertinent when the primary tumour is *in situ*, as tumour response within the prostate appears less profound compared to that observed for metastatic disease (66, 67). Further studies will need to investigate the immune infiltration profile associated with androgen deprivation, as well as the potential impact of anti-inflammatory therapies on local tumour response.

Online methods and raw data

The code used to conduct the analyses is available at https://github.com/stemangiola/ADT_fat. The sequenced reads raw files are available at <https://ega-archive.org/> with the identifier EGAD00001004971.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/EC-19-0029>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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