Evolutionary selection of alleles in the melanophilin gene that impacts on prostate organ function and cancer risk

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Received 09 March 2021; revised version accepted 03 September 2021;

ABSTRACT

Background and objectives: Several hundred inherited genetic variants or SNPs that alter the risk of cancer have been identified through genome-wide association studies. In populations of European ancestry, these variants are mostly present at relatively high frequencies. To gain insight into evolutionary origins, we screened a series of genes and SNPs linked to breast or prostate cancer for signatures of historical positive selection.

Methodology: We took advantage of the availability of the 1000 genome data and we performed genomic scans for positive selection in five different Caucasian populations as well as one African reference population. We then used prostate organoid cultures to provide a possible functional explanation for the interplay between the action of evolutionary forces and the disease risk association.

Results: Variants in only one gene showed genomic signatures of positive, evolutionary selection within Caucasian populations melanophilin (MLPH). Functional depletion of MLPH in prostate organoids, by CRISPR/Cas9 mutation, impacted lineage commitment of progenitor cells promoting luminal versus basal cell differentiation and on resistance to androgen deprivation.

Conclusions and implications: The MLPH variants influencing prostate cancer risk may have been historically selected for their adaptive benefit on skin pigmentation but MLPH is highly expressed in the prostate and the derivative, positively selected, alleles decrease the risk of prostate cancer. Our study suggests a potential functional mechanism via which MLPH and its genetic variants could influence risk of prostate cancer, as a serendipitous consequence of prior evolutionary benefits to another tissue.

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BACKGROUND AND OBJECTIVES

Several hundred inherited gene variants or SNPs impacting on cancer risk have been identified via genome-wide association studies (GWAS). Their individual impact on cancer risk is very modest but they are present at relatively high frequencies in European descent (risk allele frequency >5%; odds ratio < 1.5) [1]. Although the causal variants are in many cases unidentified, the functions they regulate suggest that some contribute by being active in cancer cells and perhaps epistatic with acquired mutations [2]. Approximately one-third of those cancer-associated variants have been shown to be pleiotropic for multiple cancers [1]. An example is given by telomeres-related loci at the TERT-CLPTM1L region associated with lung, bladder, prostate and cervical cancer risk [3]. Some important pleiotropic variants include those within MYC, TERT and HNF1B, linked to different cancer types [4].

Deaths from cancer are mostly post-reproductive and are therefore provided, at best, with only weak evolutionary selective pressure [5]. Alleles with a small impact on risk of cancer, unchecked by natural selection, could therefore increase, over time by drift, founder effects or antagonistic pleiotropy in which a trait selected for a fitness benefit, carries a post-reproductive trade off via another trait [6]. Along similar lines, a gene variant might be positively selected in one historical, environmental context but imparts a risk, including cancer, in another contemporary context. This represents an evolutionary mismatch [7] and might be applied to alleles selected 5000–10 000 years ago for skin depigmentation, associated with risk of UVB exposure and linked to skin cancer [8].

Aside from the gene variants linked to skin cancer risk, there are not many examples to date of inherited variants that are associated with cancer risk and detected SNPs that have been positively selected. The TP53 binding domain of the Kit ligand regulatory domain, associated with testicular cancer, was reported under positive selection [9] but this finding has been disputed [10]. Strong signature of natural selection was also reported at the FHIT locus that predisposes to prostate cancer [11].

Gene variants in the hormonal signalling pathways or other critical functions of breast and prostate organs might be candidates for positive selection and impact on cancer risk; some prior evidence supports this notion. African black men and Afro-American men show an increased risk of prostate cancer and have a higher frequency of androgen receptor duplications associated with adverse cancer progression [12]. An epidemiological study [13] suggested that variants within BRCA1 may have been positively selected for a fecundity benefit. Specific BRCA1 mutations are present at relatively high frequencies in certain populations but this has been plausibly ascribed to historical founder effects rather than selection [14].

In the light of these uncertainties, we carried out a proof of principle study to identify evolutionary positive selection in any cancer GWAS SNPs. We further sought to uncover a functional role for any such gene with cancer-associated SNPs that might explain its impact on cancer risk and its evolutionary adaptive significance.

We selected a total of 36 SNPs that were significantly linked to prostate cancer that had been fine mapped and functionally annotated [15]. Of these, 25 were SNPs within known genes and a further 11 were intergenic. Additionally, we selected a further eight genes with known critical functions in breast/prostate tissues including BRCA1/2 and oestrogen receptors genes (ESR1/2). One potential candidate for selection, the androgen receptor, could not be evaluated because analysis was compromised by its single copy X chromosome status. These genes and SNPs were interrogated using different, standard summary statistics able to detect genomic signals of positive selection in humans within an estimated window of historical, evolutionary time [16]. Our objective was to seek to identify and functionally validate individual candidates that had genomic signals of positive selection, and not to determine the overall prevalence of positive selection of cancer GWAS alleles. This would require a much larger, re-iterated study.

We found none of the SNPs or gene regions evaluated had significant evidence of positive selection with two exceptions. One was in the gene PPP1R14A associated with increased prostate cancer risk but selected within an African population and we did not follow it up functionally. The other positively selected gene encoded MLPH, the derivative allele in Caucasian populations reducing prostate cancer risk [15]. This gene has a recognized function in skin pigmentation which raises the puzzle of why it might impact on cancer within the prostate. We therefore coupled our genomic analysis with an exploration of MLPH function in the prostate using a mouse organoid system. The results suggest a plausible functional link between the selected GWAS allele and reduced prostate cancer risk and adds to our understanding of the multiple evolutionary and genetic influences on cancer.
METHODOLOGY

Genomic data

Phased genomic data from five different European ancestry populations (Utah residents of Northern and Western European ancestry, CEU; Finnish, FIN; British, GBR; Iberian Population, IBS; Tuscans, TSI) and one African population (Yoruba, YRI, used as reference) were obtained from the 1000 Genome Project Phase 3 [17]. A total of 599 individuals were analysed (Supplementary Table S1). Thirty-six SNPs and eight genes or gene clusters were scanned for positive selection (Supplementary Tables S2 and S3).

Selection analysis

The integrated Haplotype Score (iHS) and the Cross Population Extended Haplotype Homozygosity (XP-EHH) were implemented according to previous methods [18, 19] and carried out with selscan [20]. Clustering analysis was implemented to conservatively validate significant results. Linear mixed models were used to test selection strength on MLPH at different latitudes. Extended haplotype homozygosity (EHH) analysis and haplotype-bifurcation diagrams (HBD), introduced by Sabeti et al. [21], were used to evaluate which allele carries the haplotype homozygosity and thus possibly being under positive selection. Tajima’s D [22] and Fay and Wu’s H statistics were computed using the package PopGenome [24]. Statistical analyses were performed using R v3.6.1 [25]. Exhaustive description is provided in Supplementary Data.

MLPH expression analysis

MLPH expression was assessed by analysing RNA expression data from the Human Protein Atlas [26] and Gent2 databases [27]. A total of 12 655 and 62 978 RNA expression data were separately analysed from the first and latter database (Supplementary Table S4). Details are provided in Supplementary Data.

Prostate organoid culture

Mouse prostate tissue isolation and organoid growth were carried out as described by Drost et al. [28] and detailed in Supplementary Data.

CRISPR/Cas9 mutation of Mlp

To generate Mlp mutant cells, mouse prostate organoids were dissociated into single cells and transduced with lentICRISPRv2 [29] containing Mlp sgRNA. Methods are provided in detail in Supplementary Data.

Organoid functional studies

Materials and Methods describing the molecular and functional analysis including Mlp mutant organoids genotyping, western blots, co-immunoprecipitation, immunohistochemistry (IHC), MLPH gene expression in prostate organoids and organoid quantification are detailed in Supplementary Data.

RESULTS

We performed genomic scans for selection in five different Caucasian populations as well as one African reference population. We combined different standard summary statistics able to detect genomic signals of positive natural selection in an estimated window of evolutionary time [16]. We first used two complementary statistics grounded on long haplotype detection and able to identify recent episodes of selective sweeps (age <30 000 years): iHS [18] and XP-EHH [19]. We then employed two different statistics grounded on the frequency expectations of the variant under neutrality: Tajima’s D [22] (age <250 000 years) and Fay & Wu’s H [23] (age <80 000 years).

To assess the reliability of our statistical tests, we used the LCT/MCM6 region as control. We first computed the standardized absolute iHS score, (hereafter as iHS) and the standardized XP-EHH statistics for every SNP in the LCT/MCM6 region and within each single population. Results (Supplementary Fig. S1) showed clusters of significant iHS and XP-EHH values consistent with scientific literature advocating positive selection at LCT/MCM6 region [30]. The SNPs rs4988235 and rs182549, associated with the lactase persistence phenotype [30], as expected, show high and significant iHS scores in CEU, GBR, FIN and IBS, although in the latter dropping significance after correcting for multiple testing (Supplementary Table S5). In contrast, the distribution of iHS values in the Tuscan population does not support any selective sweep even for rs4988235 and rs182549. These two last SNPs are missing in the reference YRI dataset and therefore not analysed with XP-EHH. The graphical assessment approach, supported by EHH and HBD, suggest the derived alleles carrying the haplotype homozygosity and thus being the target of selection (Supplementary Fig. S2A) as previously demonstrated [31]. The two neutrality tests, Tajima’s D and Fay and Wu’s H do not provide support for positive selection [31] (Supplementary Fig. S2B) which agree with previous claims on positive selection occurring within the past 5000–10 000 years [31]. Past reports showed no evidence of selection in CEU using the same neutrality tests [32].

Selection in Europeans for the MLPH locus involved in prostate cancer susceptibility

The overall results are shown in Supplementary Tables S6–S8. For the majority of these genetic markers, there was no evidence for positive selection. Only MLPH showed strong evidence for positive selection in European populations. One other gene, PPP1R14A, had strong
MLPH is expressed at high levels in prostatic tissue

To further investigate the role of MLPH, we queried MLPH RNA expression datasets available within the Human Protein Atlas [26] (GTEx dataset) and Gent2 web databases [27]. Despite MLPH being involved in melanosome transport, the human tissue that expresses the highest levels of this gene is, on average, the prostate followed by salivary glands and stomach, while MLPH is expressed at a much lower level in the skin (Supplementary Fig. S7, top). A similar trend is observed in cancer tissues, where prostate cancer expresses the highest levels of MLPH, followed by melanoma and pancreatic cancer (Supplementary Fig. S7, bottom). We found no significant differences between the expression of MLPH in healthy prostate tissue and prostatic cancer (P value: 0.1425; Fig. 3A). A comparison between the expression of MLPH between two healthy tissues, skin and prostate showed a significantly higher level of expression in the latter (P value < 2.2 × 10⁻¹⁶; Fig. 3B). When we compared the expression of MLPH in skin comparing sun exposed versus sun protected skin, we found a significantly higher level of expression in the former (P value: 0.007774; Fig. 3C).

Functional impact of MLPH loss in prostate tissue

Protein expression analysis in benign and prostate cancer samples showed that tissues that contained the risk SNPs expressed lower levels of MLPH [35]. To investigate the mechanism by which MLPH contributes to prostate cancer risk, we performed functional studies in prostate organoids, 3D in vitro models of prostate epithelia homeostasis and function [36]. Cells within these in vitro cultures, derived from adult mouse prostate, differentiate and organize into the major cell types seen in the adult gland; inner luminal cells surrounded by basal cells.

We generated genetically modified prostate organoid clones using CRISPR/Cas9 and a sgRNA targeting a region of exon 3 of the mouse Mlph gene that codes for the RAB27a-binding domain. Sequencing genomic DNA from the organoid clones identified frameshift mutations in both Mlph alleles at the sgRNA target site.
**Figure 1.** Selection on rs11891348 and rs11891426 identified by iHS and XP-EHH. The iHS scores are expressed as absolute value. Lines indicate iHS or XP-EHH scores for each SNP tested. The red dashed line marks the significant threshold. The genomic position of the SNP rs11891348 is indicated by the blue vertical line. The green vertical line indicates the genomic position of rs11891426. Red dot: rs11891348; brown dot: rs11891426.

**Figure 2.** Selection strength acting on MLPH at different latitudes. Distribution of absolute iHS (left) and XP-EHH (right) scores for each European population. The dashed line represents a linear regression for trend with 95% of confidence interval (grey shadow).
Mlph-specific RNA transcripts were found in the mutant organoids suggesting that they did not undergo nonsense-mediated decay (Supplementary Fig. S8E–G). We also identified MLPH protein which may reflect the common event found in genes with CRISPR-induced frameshifts due to alternative splicing (Supplementary Fig. S8D) [37]. To confirm the generation of homozygous mutant clones, we analysed the Mlph mutant transcripts and found only mutant sequences at the target site (10/10 sequenced transcripts). Co-immunoprecipitation assay using MLPH and RAB27A antibodies was carried out to investigate if the protein seen in the mutant clones was functional. Western blot analysis on lysate from control and mutant organoid samples immunoprecipitated with MLPH antibody showed that RAB27A was bound to control but not to mutant MLPH protein (Fig. 4C). Actin, which is part of the melanosome transport complex, was also found to be preferentially associated with control and not mutant MLPH. These assays therefore show that we have generated prostate organoids that express mutant versions of MLPH protein that are functionally deficient.

Microscopic analysis of the mutant Mlph 3D cultures showed the presence of a higher number of organoids with lumens (Fig. 4A). Mlph mutants, tested by IHC assays, showed an increase in inner luminal cells (CK8 staining) and a decrease in outer basal cells (p63 staining) (Fig. 4B). This result was confirmed by qRT-PCR where Mlph mutants showed a significant increased expression of CK18, a luminal marker (P values <0.05) and a decreased expression of basal markers (Trp63 and CK5; P values <0.05) (Fig. 4D). Mutant Mlph organoids showed the consistent presence of structures with no basal cells, which were not observed in control organoids. Luminal-only organoids are a property of prostate cancer cells compared to normal prostate and have been proposed to be a source of committed luminal progenitors that promote adenocarcinoma when grafted into mice [38]. Quantification of CK8 and p63 IHC staining confirmed that Mlph mutant samples contain more organoids with lumen, and include those that do not contain basal cells (Fig. 4E–G). Although we observed a reduction in the number of Mlph mutant organoid formation (about 49% decrease, Fig. 4H), cell viability assays on the organoid cultures revealed a slightly higher level of growth in the mutant (Fig. 4I). Further analysis showed mutant organoids were bigger than controls, in particular the ones containing lumens (Fig. 4J). The reduction in organoid formation may be due to the lower number of basal cells found in the mutant as these cells have been shown to be more efficient at forming organoids compared to luminal cells [36]. Therefore, our data are consistent with the loss of Mlph leading to an increase in luminal cells giving rise to larger lumen containing organoids.

MLPH functional loss promotes luminal cell resistance to androgen deprivation therapy

Prostate cells are dependent on the male hormone, androgens, and in the clinic, androgen withdrawal is the first line of therapy for prostate cancer patients. Inhibition of this pathway in patients is obtained using second-generation AR inhibitors such as enzalutamide. To investigate the response of Mlph mutant prostate cells to androgen deprivation, we treated organoid cultures with DHT, no DHT and no DHT plus enzalutamide (Fig. 5A and B). Mlph mutants and controls had similar sensitivities to androgen deprivation in cell viability assays with enzalutamide having a markedly higher effect on organoid growth than lack of DHT for both (Fig. 5D). Differences were observed in the type of organoids growing in these conditions. In control
Figure 4. *Mlph* mutant prostate organoids contain more luminal cells. (A) Brightfield images of LacZ control and *Mlph* mutant organoids. Low magnification is 2× and high magnification is 4×. (B) Haematoxylin and Eosin (H & E) stain, p63 and CK8 immunohistochemistry on sections of control and *Mlph* mutant organoids. (C) Co-immunoprecipitation of MLPH with RAB27a and ACTIN in control and *Mlph* mutant organoids. Organoid lysates were immunoprecipitated with anti-MLPH antibody followed by Western blot analysis of RAB27a and ACTIN. (D) qRT-PCR of *Trp63*, *Ck5* and *Ck18* in control and mutant organoids. Mean and SD (error bars) are indicated. (E) the percentage of basal and luminal organoids formed from control and *Mlph* mutant cells. (F) the percentage of control and *Mlph* mutant organoids with CK18-positive cells, based on antibody stains. (G) the percentage of control and *Mlph* mutant organoids with no p63 positive cells, based on antibody stains. (H) Quantitation of the number of organoids formed from control and *Mlph* mutant cells. The number of organoids were counted from 4× images from four wells of each organoid line. (I) *Mlph* mutant and control organoid fold growth after seven days culture based on CellTiter Glo cell viability assay. (J) *Mlph* mutant and control organoid diameter. The significance of the data was analysed using a Student’s t-test, and differences between two means with a *P* value <0.05 were considered significant. Error bars in the graphics represent the standard error of the mean.
cultures, both enzalutamide treatment and lack of DHT gave rise to mostly organoids without lumens, with solid basal type organoid growth being promoted (compare LacZ4 and LacZ6 cultures in Fig. 5C to 4E). In contrast, in the Mlph mutants luminal type organoids were present in both androgen deprivation treatments. The difference was particularly prominent in the enzalutamide-treated cultures as lumen containing organoids were totally absent in control samples (Fig. 5C). IHC staining for the basal cell marker p63 within mutant samples (DHT treated and no DHT plus enzalutamide) showed enzalutamide-treated samples being enriched with luminal-only organoids (Fig. 5E). These data show that loss of Mlph function leads to the increased survival of prostate luminal cells in androgen deprivation conditions. To further investigate the relationship between MLPH and AR signalling, we analysed the expression of MLPH in prostate organoids grown without DHT and in the presence of enzalutamide. MLPH expression was found to be reduced in the enzalutamide treated sample compared to controls grown in DHT (Fig. 5F). Androgen target gene expression analysis on mutant MLPH organoids showed an increase in mutant samples compared to control samples in some target genes, namely Nkx3.1 and Fkbp5, but not others, such as Ar and Pmep1a (Fig. 5G).

**DISCUSSION**

In the present study, we dissected the genetic architecture of a limited set of loci associated with breast/prostate cancer susceptibility to determine if any had signatures of positive selection. Our analysis showed no evidence of selection in almost all cases. We conclude that the high frequency of these allelic variants in European populations most likely reflects neutral drift or founder effects. In contrast, we found strong evidence for recent positive selection in MLPH for two common SNPs rs11891426 [35] and rs11891348 [15] as well as for the whole MLPH gene. A recent study has also found signatures of positive selection in MLPH in the CEU population [39]. When we explored which of the two alleles underwent selective sweeps, we found that in both SNPs, the derived and protective T alleles were the ones with long-range linkage disequilibrium at the core SNP. The T alleles of both SNPs are therefore significantly correlated. Although a long-range haplotype carrying the derived TT alleles is present in high frequency in all European populations examined here, it is worth noting the presence of different really low frequency long range haplotypes carrying both ancestral and derived alleles at the core haplotype (Fig. 1 and Supplementary Fig. S5B). This haplotype diversity might suggest the occurrence of few recombinations and thus probable recent and/or ongoing selection.

The historical, adaptive logic of a positively selected allele can be difficult to determine. The MLPH alleles we found to

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**Figure 5.** Mlph mutant organoids have an increased number of AR-independent luminal cells. Brightfield images of control and Mlph mutant organoids grown with DHT or (A) no DHT, and (B) no DHT and 10μM enzalutamide. (C) The percentage of basal and luminal organoids formed from control and Mlph mutant prostate cells grown in no DHT or no DHT and 10μM enzalutamide. (D) survival fraction of control and Mlph mutant cells grown with DHT, no DHT or no DHT and 10μM enzalutamide as assayed by CellTiter Glo viability assay. Each sample normalized to DHT growth. (E) The number of control and Mlph mutant organoids grown in DHT or in no DHT and 10μM enzalutamide with no p63 stain. (F) qRT-PCR of Mlph in control organoids grown in DHT, no DHT, or no DHT and 10μM enzalutamide. Mean and SD (error bars) are indicated. (G) qRT-PCR of Ar, Nkx3.1, Fkbp5 and Pmep1a in control and Mlph mutant organoids. Mean and SD (error bars) are indicated.
carry the long-range haplotype, reduce the risk (OR: 0.9) of prostate cancer but, as lethal prostate cancer is almost entirely post-reproductive, it is very unlikely that cancer itself provided the selective pressure or adaptive advantage [5]. This most likely derived from an impact of MLPH alleles on skin pigmentation responses.

The MLPH gene encodes for the MLPH protein involved in melanosome transport [33]. The MLPH protein forms a ternary complex with the small Ras-related GTPase Rab27a and the motor protein myosinVa (Rab27a-Mlph-MyoVa) where MLPH acts as a tether to enhance the transfer of melanosomes from melanocytes to the adjacent keratinocyte providing pigment needed for hair, skin and eye colouring [40]. In humans, mutations in each of the above three genes lead to different types of Griscelli syndrome, a hypopigmentation disorder [41]. When skin is exposed to the sun, the expression of MLPH is significantly higher than the sun-protected skin suggesting MLPH expression associated with UV irradiation response. Transfer of melanosomes from melanocytes to keratinocytes enables darker pigmentation and plays an essential role in protecting the skin from UV irradiation [42].

Higher expressed MLPH is therefore associated with darker phenotype in sheep [43] and rabbits [44]. In Europeans, pigmentation phenotypes were found to reflect the geographic allocation, lighter in the north and darker in the south [45]. We found a north-south latitudinal cline for selection strength acting on MLPH, possibly reflecting evolutionary adaptation to latitude-dependent UV levels and a role in skin darkening/tanning might be plausible. The stronger selection pressure acting in the south may reflect an evolutionary drive for higher UV protection, which incidentally impacted prostate cancer risk. Sun exposure was found to inversely correlate with prostate cancer incidence [46] and a past research showed a north-south trend for prostate cancer mortality, with lower rates in the south with higher UV levels [47]. Environmental conditions related to different UV levels might have exerted on European ancestors a selective pressure and likely those TT (rs11891426 and rs11891348) carriers might have had a stronger response to the sun exposure.

Despite its function in skin, MLPH is not exclusively expressed in the skin, but is highly expressed in healthy and malignant prostate tissue. Within prostatic tumours, lower levels of MLPH are associated with more aggressive disease [48]. Similarly, in breast cancer, the more malignant oestrogen negative tumours express lower levels of MLPH than oestrogen positive tumours implying hormonal regulation. Rs11891426 protective allele carriers express higher MLPH levels in prostate tumours [35].

Our functional studies on MLPH loss in cultured organoids of mouse prostate tissue suggest a possible rationale of the impact of MLPH alleles on prostate cancer risk and aggressiveness. Loss of MLPH function in prostate organoids promotes luminal differentiation and growth even in androgen deprivation conditions. In addition, our studies show the presence of luminal-only structures specifically in mutant cultures, which are more resistant to enzalutamide treatment. These structures have been found to contain luminal precursors and are preferentially found in cultures from aggressive prostate tumour tissue with loss of tumour suppressors Tp53 and Pten [38]. Studies in mice have shown that luminal cells are more sensitive to neoplastic transformation by the loss of Pten than basal cells of the prostate [49].

The risk allele for MLPH expresses lower levels of protein and one possibility is that this, consistent with our Mlph mutant studies, increases the number of luminal progenitor/stem cells—the likely target cell population for drivers of clinical prostate cancer [50], which is mostly luminal in phenotype. The selected SNP alleles reside within the androgen-binding site of MLPH. Consistent with this, we did observe a decrease in MLPH expression in prostate organoids following androgen deprivation therapy. Lack of MLPH led to changes in the expression of some androgen target genes. Therefore, our data suggest an interplay between androgen signalling and MLPH expression and a luminal phenotype, which may change with neoplastic transformation and patient treatment.

SUPPLEMENTARY DATA
Supplementary data is available at EMPH online.

ACKNOWLEDGEMENTS
The authors thank Mitsunori Fukuda for his anti Slac2-a antibody and Professor Ros Eeles for data summaries on SNP acquisition in prostate cancer and breast cancer, respectively.

AUTHOR’S CONTRIBUTIONS
L.E. analysed the data, carried out the statistical and bioinformatics analysis, interpreted the results and contributed to devise the study. J.C.F. performed the functional study with contribution from G.S.R and J.N. A.S designed, supervised and interpreted the functional experiments. M.G. conceived and planned this study, interpreted the results and contributed to devise the study. J.C.F. performed the statistical and bioinformatics analysis, interpreted the results and contributed to devise the study. L.E. and M.G. wrote the manuscript with contributions from A.S. and input from all authors.

FUNDING
This work was supported by a Wellcome Trust award to the Centre for Evolution and Cancer (105104/Z/14/Z) and The Institute of Cancer Research, London (M.C.). J.C.F. and G.S.R were funded by a Prostate Cancer UK grant (RIA17-ST2-01).

Conflict of interest: None declared.
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