Contributions to Drug Resistance in Glioblastoma Derived from Malignant Cells in the Sub-Ependymal Zone

Sara G.M. Piccirillo1, Inmaculada Spiteri2, Andrea Sottoriva2,3, Anestis Touloumis2,4, Suzan Ber1, Stephen J. Price5, Richard Heywood1, Nicola-Jane Francis6, Karen D. Howarth7, Vincent P. Collins8, Ashok R. Venkitaraman9, Christina Curtis9, John C. Marioni9, Simon Tavare2, and Colin Watts1,5

Abstract
Glioblastoma, the most common and aggressive adult brain tumor, is characterized by extreme phenotypic diversity and treatment failure. Through fluorescence-guided resection, we identified fluorescent tissue in the sub-ependymal zone (SEZ) of patients with glioblastoma. Histologic analysis and genomic characterization revealed that the SEZ harbors malignant cells with tumor-initiating capacity, analogous to cells isolated from the fluorescent tumor mass (T). We observed resistance to supramaximal chemotherapy doses along with differential patterns of drug response between T and SEZ in the same tumor. Our results reveal novel insights into glioblastoma growth dynamics, with implications for understanding and limiting treatment resistance. Cancer Res; 75(1); 1–9. ©2014 AACR.

Introduction
The basis of phenotypic diversity and treatment failure in human glioblastoma is poorly understood. Murine models of gliomagenesis point to sub-ependymal neural stem cells (NSC) as a putative cell of origin for astrocytic tumors. The stepwise premalignant loss of tumor suppressors p53, NF1, and PTEN (1, 2) has been shown to lead to the development of an aggressive disease characterized by resistance to genotoxic injury (3).

In addition, stratifying patients using transcriptional profiles derived from a large cohort of glioblastoma single-tumor samples (4) has identified multiple disease subtypes, which may have prognostic significance (3, 6). However, emerging data on genomic intratumor heterogeneity in glioblastoma indicate spatial segregation of genetically distinct clones in the same tumor (7), making the interpretation of single-sample tumor data challenging. Importantly, this may contribute to the pervasive failure of treatment in patients with glioblastoma.

Clinical trials have established that use of a fluorescence biomarker, 5-aminolevulinic acid (5-ALA), can enhance the surgical resection of glioblastoma (8). We have demonstrated the use of 5-ALA in a fluorescence-guided multiple sampling (FGMS) strategy that permits real-time spatially segregated tumor sampling during surgery (7, 9). Combining visible fluorescence with neuroanatomy allows for the objective distinction of the tumor mass T (visible fluorescent). Importantly, a spatially distinct and visibly fluorescent sub-ependymal zone (SEZ) can also be identified in a subset of patients with glioblastoma.

Here, we report an integrated genomic analysis of SEZ and T samples, obtained by FGMS, which reveals that malignant cells in the SEZ contribute to tumor growth. Functional characterization confirms that the SEZ contains tumor-initiating cells (TIC) that can recapitulate the disease in orthotopic patient-derived xenograft models in a manner similar to TICs isolated from the corresponding T. TICs in the SEZ contribute to resistance to chemotherapy and show differential patterns of response when compared with T of the same patients.

Materials and Methods
Glioblastoma sample collection
Patient informed consent was obtained through our research clinic (10). Tissue collection protocols were compliant with the UK Human Tissue Act 2004 (HTA Licence ref 12315) and approved by the Local Regional Ethics Committee (LREC ref: 04/Q0108/60). No difference in 5-ALA labeling capacity was observed among patients. See Supplementary Experimental
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Procedure for details on 5-ALA administration and sample collection.

Quantitative real-time PCR analysis
Total RNA was extracted from T and SEZ tissues using TRIzol (Invitrogen) according to the manufacturer’s instructions. RNA was treated with DNase (Qiagen) and cDNA was synthesized from 5 μg of total RNA using Random Primers (Invitrogen), and a Superscript III First-Strand Synthesis System for real-time PCR (RT-PCR; Invitrogen). The RT-PCR for Nestin, Gfap, Sox2, and MIB1 transcripts was performed using CFX96 RT-PCR (Bio-Rad), RT² qPCR Primer Assay and SYBR Green Master Mix (Qiagen) according to the manufacturer’s instructions. 18S was used as the housekeeping reference. Relative expression quantification was performed by the ΔΔCt method. Experiments were performed in triplicate and each experiment was repeated three times.

Cell line derivation and implantation
Cell cultures from T and SEZ of 42 patients were established from patients with glioblastoma undergoing surgery at Addenbrooke’s Hospital, Cambridge, in 2010 to 2012. The cells were isolated as described previously (9, 11, 12) and used either uncultured (primary) or propagated (brieﬂy cultured) in serum-free medium. The U87 cell line was obtained from the ATCC, cultured according to the supplier’s recommendations and used just after resuscitation. Tissue collection to establish HFNSCs was approved by the Local Regional Ethics Committee. Cells were established in 2011 and grown in serum-free medium to form neurospheres and used at early passage. All the cell cultures have been tested for Mycoplasma contamination by PCR before use. See Supplementary Experimental Procedure for further details.

DNA and RNA extraction
DNA from T and SEZ tissues of 14 patients with glioblastoma was extracted for copy-number analysis using the DNeasy Blood and Tissue Kit (Qiagen). RNA from T and SEZ tissues of 15 patients with glioblastoma was extracted for gene-expression analysis using TRIzol (Invitrogen) and cleaned up using MiniElute columns (Qiagen). See Supplementary Experimental Procedure for details on copy-number and gene-expression analysis. Copy-number results were validated by FISH as described in the Supplementary Experimental Procedure.

Drug treatment assay
Treatment with temozolomide, cisplatin, and cediranib was evaluated using the in vitro BrdUrd cell proliferation assay (Millipore). A total of 3 × 10³ cells were plated in triplicate per treatment condition. Control wells for temozolomide, cisplatin, and cediranib are shared as these treatments have been applied in the same experiments. One day after plating, the treatment was applied for 3 days. BrdUrd was applied in the final 24 hours of the treatment. Each experiment was repeated three times. See Supplementary Experimental Procedure for details on drug concentration.

MGMT promoter methylation
Analysis of MGMT promoter methylation was performed by PCR (13) and by pyrosequencing (14). In brief, DNA was bisulfite converted and subsequently subjected to PCRs using speciﬁc primer pairs for the methylated or the modiﬁed unmethylated DNA. PCR products were resolved in a 2% agarose gel stained with SybrSafe. See Supplementary Experimental Procedure for further details.

Phylogenetic reconstruction
Multiple spatially separated samples taken from each glioblastoma were collected to reconstruct the phylogenetic relationship between the tumor and the SEZ using copy number proﬁles and molecular clock analysis. See Supplementary Experimental Procedure for further details.

Statistical analysis
For in vivo experiments using Nod/Scid animals, we performed Kaplan–Meier survival analysis using the log-rank test for hypothesis testing.

For drug response analysis using BrdUrd, a one-way ANOVA was performed for each patient.

The P values for the pairwise mean comparisons of each treatment with the corresponding control were calculated using the Tukey Honestly Signiﬁcant Difference test. In the related ﬁgures and for each patient, we plotted the normalized mean treatment response using the corresponding mean control as a reference, except in Supplementary Fig. S12, where we normalized to the mean of the 50 μmol/L temozolomide treatment response. The minimum and maximum normalized values are provided to present the dispersion of the normalized data.

Results
SEZ harbors residual disease in patients with glioblastoma
We screened 65 patients with glioblastoma given 5-ALA, and conﬁrmed that visibly ﬂuorescent disease extended to the SEZ in 65% (42/65) of the cases. Multiple samples were obtained from 14 patients. Histologic features of high-grade glioma (15) were detected in SEZ and T of the patients (Fig. 1A; additional two representative glioblastomas are shown in Supplementary Fig. S1A and S1B). SEZ tissue was also used to identify the ependymal layer and conﬁrm correct sampling (Supplementary Fig. S2).

Glioblastoma has been shown to heterogeneously express glial fibrillary acidic protein (Gfap; ref. 16), and consistent with this, we noted high expression of Gfap in SEZ samples compared with matched T samples (Fig. 1B and C top, and Supplementary Fig. S1C). Expression of the precursor marker Nestin was detected both in T and SEZ tissues (Fig. 1B and C middle, and Supplementary Fig. S1C). The SEZ showed increased vascularization in comparison with T, as determined by CD31 expression (Fig. 1B and C, bottom). Weak expression of the neuronal marker Tuj1 was observed in both areas (Supplementary Fig. S1C). One of the histologic hallmarks of glioblastoma is a high proliferative activity (17), and we observed similar numbers of mitotic cells in T and SEZ (MIB1 index 20.5 ± 2.8 and 18.1 ± 1.9, respectively; Supplementary Fig. S1C). The extent of focal necrosis was similar in T and SEZ of the same patients (except for sp40; Supplementary Table S1). Consistent with immunohistochemical analysis, T and SEZ contained similar amounts of tumor tissue (except for sp54; Supplementary Table S1). Together, these data suggest that regulatory mechanisms promoting proliferation and vascularization are common to the T and the SEZ tissues.

To conﬁrm these ﬁndings, we performed real-time analysis of gene expression for markers of glial and precursors cells (Gfap and...
Nestin), stem cells (Sox2), proliferation (MIB1) on T and SEZ tissues of three glioblastomas in total (Supplementary Fig. S3). Our results show increased expression of Gfap in the SEZ compared with the corresponding T (Supplementary Fig. S3). The expression of Nestin and Sox2 is similar between T and SEZ, except in sp10 for Sox2 (Supplementary Fig. S3).

We applied high-throughput genomic profiling techniques to the SEZ and T to characterize each of these regions. DNA/RNA were extracted from the tissues of the 14 patients (Supplementary Table S2A). After quality assessment, we analyzed copy-number aberrations (CNA) of 8 patients and gene-expression profiles of 9 patients (6 of whom were common to both analyses; Supplementary Table S2B) shows the patient clinical information. Our data reveal multiple aberrations (e.g., EGFR amplification and CDKN2A deletions) common to T and the corresponding SEZ in individual patients (Fig. 2A and Supplementary Fig. S4). In 6 of 8 patients, the SEZ had an equal or smaller number of putative driver aberrations with respect to the corresponding T (mean difference in the number of aberrations is 1.27 ± 0.38; P value = 0.12). CNA results were validated by FISH for three glioblastoma drivers, EGFR, MET, and PTEN (4), in 3 patients with available tissue (Fig. 2B and Supplementary Fig. S5A–S5H). Because we observed in vitro aberrations in the corresponding cell cultures (Supplementary Fig. S6), we restricted further genomic analysis to tissue samples.

Clustering of the gene-expression data (18) revealed that only three of nine paired samples (SEZ and T from the same patient) clustered tightly together, whereas five of nine SEZ were assigned to the same subcluster, suggesting a SEZ-specific expression profile across patients (Fig. 2C).

We used a previously published classifier to assign our samples into one of four glioblastoma subtypes (6). Seven of nine SEZ were classified as mesenchymal (sp41, sp42, sp52, sp54, sp56, sp57, and r4), and the remaining two were classical (sp49 and sp55). For 6 of 9 patients, the SEZ was assigned to a different subtype than the corresponding T. T samples were distributed amongst the four subtypes (classical, mesenchymal, neural, and proneural; Fig. 2D).

We next investigated whether gene-expression levels differed between SEZ and T. We rejected the hypotheses of no significant differences in the expression levels of all genes between SEZ and T (P value < 0.0001), which suggests that there are differentially expressed genes in SEZ and T. We next used the R package HDTD to identify gene ontology (GO) terms whose genes are differentially expressed (Supplementary Table S3).

TICs reside in the SEZ

We next extended the characterization of T and SEZ to TIC populations. In neuroepithelial malignancies, the purification of TICs remains challenging because no robust cell-surface marker has been identified to distinguish tumorigenic and nontumorigenic cells (19, 20). Initial data identifying CD133/Prominin1 as a marker in human glioblastoma (21) were subsequently challenged both in primary and cultured TICs (reviewed in ref. 19). More recently, the cell surface marker CD15/SSEA-1 has been identified as a possible TIC marker in glioblastoma and medulloblastoma (22, 23). CD15 is a carbohydrate moiety expressed by neural stem and progenitor cells (24), but its use as glioblastoma marker did not find additional confirmation (25).

We therefore used a marker-independent approach (9, 11, 12, 26) to isolate cells from T and SEZ under serum-free conditions in vitro (Fig. 3A). Growth curve analysis and limiting-dilution assays confirmed long-term self-renewal and expansion (Fig. 3B and Supplementary Fig. S7), clonogenicity, and multipotency similar to the corresponding T cells (Supplementary Fig. S8).

We evaluated the expression of the precursor marker Nestin and other putative TIC markers (A2B5, CD133, and CD15) from T and SEZ (reviewed in ref. 19) and found similar expression of Nestin in T and SEZ cells, in agreement with the data in Fig. 1B and C. In contrast, A2B5-, CD133-, and CD15-positive cells were rarely found (Supplementary Fig. S9A and S9B).
Despite phenotypic differences in vitro, orthotopic inoculation of T and SEZ cells in NOD/SCID mice consistently generated tumors in all cases (Fig. 3C and D and Supplementary Fig. S10). Nonetheless, a statistically significant shorter survival was observed for animals injected with T cells compared with those injected with SEZ cells (Fig. 3C and Supplementary Fig. S10; \( P < 0.05 \)).

To our knowledge, this is the first time that SEZ cells from patients with glioblastoma have been tested for their tumorigenic potential. We analyzed the in vivo properties of enriched SEZ cells from three additional glioblastomas. In all cases, SEZ cells gave rise to tumors with similar patterns of growth and infiltration to those generated from TICs isolated from T, under the same experimental conditions (Supplementary Fig. S11A–S11C).

**TICs from the SEZ contribute to drug resistance**

The SEZ and T contained self-renewing TICs suitable for chemoresponse assays. These cells are grown in conditions that better preserve the genotype of the original disease (27) and have been proposed for use in high-throughput drug screening (28, 29). We tested the effects of the oral alkylating agent temozolomide, the current standard of care in patients with glioblastoma (30). To facilitate analysis, we assayed methylation in the promoter region of \( MGMT \), a methyltransferase that inhibits the cytotoxic effect of temozolomide and is a predictive biomarker in glioblastoma (31).

We initially treated TICs isolated from T and SEZ of 7 patients with glioblastoma with temozolomide at maximum concentrations reported for the brain and plasma (50 \( \mu \)mol/L; refs. 32, 33), but no significant treatment response was observed relative to the corresponding controls (vehicle only). Only sp12 showed a significant response in both T and SEZ (Supplementary Fig. S12). We therefore analyzed a set of TICs isolated from T of 20 patients using a dose-escalation strategy ranging from 50 \( \mu \)mol/L to 2.5 mmol/L of temozolomide, and found that only 20% of the samples exhibited a significant response at \( \geq 500 \mu \)mol/L (Supplementary Fig. S13A and S13B).
On the basis of these results, we performed cell proliferation assays for temozolomide concentrations between 50 μmol/L and 2.5 mmol/L on TICs isolated from T and SEZ of the same tumor for 8 patients. We noted that TICs continued to proliferate in the SEZ and T at supramaximal drug concentrations. We also observed that the response varied between T and SEZ of the same glioblastoma (Fig. 4A and Supplementary Fig. S14A). Three patterns emerged: differential response between T and SEZ (e.g., sp13, sp20, sp37, and sp42), both T and SEZ respond (e.g., sp17 and sp23), neither T nor SEZ respond (e.g., sp14, except at 2.5 mmol/L temozolomide for T, and sp52).

To test whether resistance to temozolomide was MGMT-dependent, we analyzed the DNA methylation status of the MGMT promoter by pyrosequencing (Supplementary Table S4) and methylation-specific PCR (Supplementary Fig. S15; ref. 13), and we found that the results were in agreement except for sp42.

Our PCR analysis revealed that four of seven paired TICs (sp14, sp23, sp37, and sp52) did not show methylation of the MGMT promoter in either the T or the SEZ consistent with the poor response to temozolomide, except for sp23 (T and SEZ) and sp37T. In contrast, sp17, sp20, and sp42 are methylated and showed a better response to temozolomide among all the tested TICs with the exception of sp20SEZ and sp42T (Fig. 4A and Supplementary Fig. S14A). All together, our results suggest that MGMT methylation status is homogeneous in T and SEZ of the same patients and generally predicts response to temozolomide (Supplementary Table S5).

We also evaluated the antimitotic agent cisplatin, previously used in glioblastoma therapy, and cediranib, an antiangiogenic inhibitor of VEGFRs with additional activity against PDGFRs, recently used in clinical trials (Fig. 4B and Supplementary Fig. S14B; refs. 34, 35). Although antiangiogenic therapies target the endothelial compartment, it has been reported that VEGFRs are enriched on the surface of TICs from glioblastoma (36). VEGF signals via its endothelial tyrosine kinase receptor 2 (VEGFR2; ref. 35), so we first confirmed that this receptor is expressed in TICs from T and SEZ (Supplementary Fig. S16). We next quantified the expression of VEGF and PDGF receptors. No significant difference was present between T and SEZ (no VEGFR1 nor VEGFR3 probe was available in the Illumina arrays; Supplementary Table S6).

Exposure to cisplatin and cediranib revealed resistant TICs in T and SEZ together with a heterogeneous response profile (Fig. 4B and Supplementary Fig. S14B). Whereas sp17 and sp23 showed sensitivity to these treatments in T and SEZ, a significant response was observed only in one of the two regions for sp13, sp20, and sp52 (Fig. 4B).

The drug response profile of TICs from T and SEZ of the same patients emphasizes their potential utility in drug development compared with standard glioma cell lines, for example, U87 and human fetal NSCs (HFNSC). U87 significantly responded to treatment with temozolomide, cisplatin, and cediranib, whereas two HFNSC lines (HFNSC and HFNSC1) were resistant to temozolomide, as previously reported (37), and to cisplatin and cediranib (Supplementary Fig. S17).
Phylogenetic reconstruction suggests different patterns of glioblastoma evolution involving SEZ malignant cells

Our genomic and chemoresponse assays data show that the SEZ harbors malignant cells that contribute to tumor growth and murine models of glioblastoma indicate that the SEZ is enriched for tumor ancestors (2, 38–40). However, this has not been confirmed in patients with glioblastoma. We analyzed SEZ and T samples to determine whether tumor cells grow out of the SEZ or into the SEZ. We reconstructed tumor ancestral trees in 8 patients, using several genomic measurements derived from multiple spatially separated samples taken from the glioblastoma mass (T1–T6) and SEZ. We have previously used this approach to describe intratumor heterogeneity in T and infer tumor evolution (7), and we now used FGMS to position the SEZ. In particular, we reconstructed phylogeny based on genome-wide DNA copy number (41). In an independent assay, we exploited the observation that cells record their ancestral history in the form of neutral DNA methylation patterns (42, 43). This analysis is not biased by the presence of non-neoplastic cells, as only highly proliferative tissues accumulate sufficient methylation events (tumors and colonic epithelium; Fig. 5A; ref. 44). We first validated the molecular clock loci chosen for this analysis (Fig. 5B and C, top) and then calculated the normal cell content in SEZ and T. The values of cellularity indicate no significant difference (Fig. 5B and C, bottom).

These orthogonal techniques yielded highly concordant phylogenies: in sp52, sp54, sp56, and sp57, the SEZ harbors tumor precursor cells that gave rise to the glioblastoma mass (Fig. 5D). In sp42 and sp49, we observed a similar trend, although the two methods are not in full agreement (Fig. 5D). Analysis of sp55 and sp58 suggests a different pattern of evolution and emphasizes the heterogeneous nature of glioblastoma.

Figure 4. TICs from SEZ contribute to drug resistance. Cells from T and SEZ of 4 patients were used to evaluate response to chemotherapeutics. A, differential response patterns to temozolomide (TMZ) were observed in T and SEZ cells of the same patients. Dose escalation has little impact on drug-resistant cells. B, distinct patterns of response are observed when the assay is performed with cisplatin and cediranib. Data are shown as normalized to control. The mean normalized absorbance as well as the minimum (−) and maximum (+) absorbance are plotted. Asterisks, statistical significance.
glioblastoma (Fig. 5D). Taken together, these data suggest that the SEZ contains a reservoir of malignant cells that are either tumor precursor clones or clones generated during glioblastoma evolution.

Discussion

Our comprehensive phenotypic, genomic, and functional analysis reveals residual disease in the SEZ of patients with glioblastoma (Fig. 1A and Supplementary Fig. S1A and S1B). TICs are present in T and SEZ of the same glioblastomas (Fig. 3C and Supplementary Fig. S10) and show differential patterns of therapeutic responsiveness and drug resistance (Fig. 4A and B and Supplementary Fig. S14A and S14B), suggesting that the SEZ should be considered as a novel potential therapeutic target in a subset of patients with glioblastoma. This is confirmed by phylogenetic reconstruction showing different patterns of tumor evolution with the SEZ harboring precursor clones or clones generated during the glioblastoma growth (Fig. 5D).

Gene-expression profiling of SEZ reveals that different glioblastoma subtypes (6) are present within the same patient. The SEZ is predominantly mesenchymal (7 patients) or classical (2 patients). T is more diverse, with representation from all four subtypes (Fig. 2D). Thus, it is possible to envisage a spectrum of expression patterns with mesenchymal/classical representing proliferative diversification at the tumor core.

Previous reports suggested that proximity to the SEZ predicts a multifocal tumor phenotype and recurrences that arise at locations distant from the initial lesion (45). More recently, it has also been reported that contact of the tumor mass with the SEZ correlates with shorter survival (46). To explore this, we isolated TICs from T and SEZ of the same patients. TICs have been described in human glioblastoma but no analysis based on FGMS has been performed. Our results show that in vivo both T- and SEZ-derived cells generated tumors; however, cells from T are more tumorigenic in comparison with the SEZ in agreement with the CNAs of the patient samples showing a trend toward a smaller number of aberrations in the SEZ. These data suggest that the tumorigenic potential is exacerbated when increased genetic alterations are acquired by the cancer genome in line with previous reports (47, 48).

Because genomic and phenotypic analyses suggest that the SEZ harbors tumor cells, it is crucial to investigate whether there are differences in response to therapy in cells isolated from this region. It has been previously shown that patient-derived TICs accurately represent parent disease (27) and have potential application in high-throughput drug screening (28, 29). Our data reveal that TICs from T and SEZ of the same glioblastomas show different patterns of response to therapies that represent the current standard of care (Fig. 4A and B, Supplementary Fig. S14A and S14B and Supplementary Table S5). This suggests that they should be targeted using different approaches. Our results also reveal that a clinically significant
fraction of cells is resistant to current treatments. This is consistent with murine data (3), indicating that multimodal stratified approaches will be essential to improve therapeutic responsiveness.

Glioblastoma evolves by following poorly understood spatial and temporal dynamics arising from cells of origin that are yet to be defined. The presence of malignant cells in the SEZ suggests two different scenarios of evolution with the glioblastoma growing into the SEZ or out of this region. Our phylogenetic data indicate that in 4 of 8 patients, a pool of malignant precursor clones evolved in the SEZ (Fig. 5D). Given the presence of NSCs in the adult human SEZ (49), it has been suggested that glioblastoma is derived from those cells (50). This concept has been investigated in mouse models (2, 38–40), but until now there has been no direct evidence of the contribution of SEZ cells in human gliomagenesis. We show that the SEZ is a reservoir of disease and could be targeted therapeutically. Consistent with this, preliminary evidence suggest that irradiation of the SEZ in patients with glioblastoma is associated with improved progression-free survival (51, 52).

In summary, we present a phenotypic, genomic, and functional analysis of residual disease in human glioblastoma (Supplementary Fig. S1A and S1B). Our approach together with FGMS provides a coherent strategy for interrogating the mechanistic basis of clinical heterogeneity in future studies. This is likely to further refine our understanding of the complex molecular landscape of glioblastoma, resulting in improved therapeutic strategies specifically aimed at targeting the SEZ.

Disclosure of Potential Conflicts of Interest
S. Tavare has provided expert testimony for Springer Verlag. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: S.G.M. Piccirillo, A.R. Venkitaraman, C. Watts
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.G.M. Piccirillo, I. Spiteri, A. Sottoriva, A. Touloumis, K.D. Howarth, V.P. Collins, A.R. Venkitaraman, C. Curtis, J.C. Marioni, S. Tavare, C. Watts
Writing, review, and/or revision of the manuscript: S.G.M. Piccirillo, I. Spiteri, A. Sottoriva, S.J. Price, V.P. Collins, A.R. Venkitaraman, C. Curtis, J.C. Marioni, S. Tavare, C. Watts
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.G.M. Piccirillo, V.P. Collins, C. Watts
Study supervision: A.R. Venkitaraman, C. Curtis, S. Tavare, C. Watts
Other (technical advice related to methodology): N.-J. Francis

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