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Author/s:
Macauda, A;Piredda, C;Clay-Gilmour, AI;Sainz, J;Buda, G;Markiewicz, M;Barington, T;Ziv, E;Hildebrandt, MAT;Belachew, AA;Varkonyi, J;Prejzner, W;Druzd-Sitek, A;Spinelli, J;Andersen, NF;Hofmann, JN;Dudziński, M;Martinez-Lopez, J;Iskierka-Jazdzewska, E;Milne, RL;Mazur, G;Giles, GG;Ebbesen, LH;Rymko, M;Jamroziak, K;Subocz, E;Reis, RM;Garcia-Sanz, R;Suska, A;Haastrup, EK;Zawirska, D;Grzasko, N;Vangsted, AJ;Dumontet, C;Kruszewski, M;Dutka, M;Camp, NJ;Waller, RG;Tomczak, W;Pelosini, M;Raźny, M;Marques, H;Abildgaard, N;Wątek, M;Jurczyszyn, A;Brown, EE;Berndt, S;Butrym, A;Vachon, CM;Norman, AD;Slager, SL;Gemignani, F;Canzian, F;Campa, D

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CANCER GENETICS AND EPIGENETICS

Expression quantitative trait loci of genes predicting outcome are associated with survival of multiple myeloma patients

Angelica Macauda[1,2] | Chiara Piredda[2] | Alyssa I. Clay-Gilmour[3] | Juan Sainz[4,5] | Gabriele Buda[6] | Miroslaw Markiewicz[7] | Torben Barington[8] | Elad Ziv[9] | Michelle A. T. Hildebrandt[10] | Alem A. Belachew[10] | Judit Varkonyi[11] | Witold Prejzner[12] | Agnieszka Druzd-Sitek[13] | John Spinelli[14,15] | Niels Frost Andersen[16] | Jonathan N. Hofmann[17] | Marek Dudziński[18] | Joaquin Martinez-Lopez[19] | Elzbieta Iskierka-Jazdzewska[20] | Roger L. Milne[21,22,23] | Grzegorz Mazur[24] | Graham G. Giles[21,22,23] | Lene Hyldahl Ebbesen[16] | Marcin Rymko[25] | Krzysztof Jamroziak[26] | Edyta Subocz[27] | Rui Manuel Reis[28,29] | Ramon Garcia-Sanz[30] | Anna Suska[31] | Eva Kannik Haastrup[32] | Daria Zawirska[33] | Norbert Grzasko[34,35] | Annette Juul Vangsted[32] | Charles Dumontet[36] | Marcin Kruszewski[37] | Magdalena Dutka[12] | Nicola J. Camp[38] | Rosalie G. Waller[38] | Waldemar Tomczak[39] | Matteo Pelosini[6] | Małgorzata Raźny[40] | Herlander Marques[29] | Niels Abildgaard[41] | Marzena Wątek[42] | Artur Jurczyszyn[31] | Elizabeth E. Brown[43] | Sonja Berndt[17] | Aleksandra Butrym[24] | Celine M. Vachon[44] | Aaron D. Norman[44] | Susan L. Slager[44] | Federica Gemignani[2] | Federico Canzian[1] | Daniele Campa[2]

[1]Genomic Epidemiology Group, German Cancer Research Center (DKFZ), Heidelberg, Germany

[2]Department of Biology, University of Pisa, Pisa, Italy

[3]Department of Epidemiology & Biostatistics, Arnold School of Public Health, University of South Carolina, Columbia, South Carolina

[4]Genomic Oncology Area, GENYO. Centre for Genomics and Oncological Research: Pfizer, University of Granada/Andalusian Regional Government, Granada, Spain

[5]Hematology department, Virgen de las Nieves University Hospital, Granada, Spain

[6]Clinical and Experimental Medicine, Section of Hematology, University of Pisa, Pisa, Italy

[7]Department of Hematology and Bone Marrow Transplantation, SPSKM Hospital, Katowice, Poland

[8]Department of Clinical Immunology, Odense University Hospital, Odense, Denmark

[9]Department of Medicine, Division of General Internal Medicine, Institute for Human Genetics, Helen Diller Family Comprehensive Cancer Center, University of California San Francisco, San Francisco, California

[10]Department of Epidemiology, Division of Cancer Prevention and Population Sciences, The University of Texas MD Anderson Cancer Center, Houston, Texas

Abbreviation: ASCT, autologous stem cell transplantation; CI, confidence interval; CTCF, CCCTC-binding factor; eQTLs, expression quantitative trait loci; GEP, gene expression profile; GWAS, genome wide association studies; HR, hazard ratio; HWE, Hardy-Weinberg equilibrium; ISS, International Staging System; LD, linkage disequilibrium; MM, multiple myeloma; OS, overall survival; PFS, progression-free survival; SNP, single nucleotide polymorphism.

Angelica Macauda, Chiara Piredda, Federico Canzian and Daniele Campa equally contributed to this work, in their respective positions.

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[11]Third Department of Internal Medicine, Semmelweis University, Budapest, Hungary
[12]Department of Hematology and Transplantation, Medical University of Gdansk, Gdansk, Poland
[13]Department of Lymphoid Malignacies, Maria Skłodowska-Curie National Research Institute of Oncology, Warsaw, Poland
[14]Cancer Control Research, BC Cancer Agency, Vancouver, British Columbia, Canada
[15]School of Population and Public Health, University of British Columbia, Vancouver, British Columbia, Canada
[16]Department of Hematology, Aarhus University Hospital, Aarhus, Denmark
[17]Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, Maryland
[18]Department of Hematology, Institute of Medical Sciences, College of Medical Sciences, University of Rzeszow, Rzeszow, Poland
[19]Complutense University, CNIO, CIBERONC, Hospital 12 de Octubre, Madrid, Spain
[20]Department of Haematology, Copernicus Memorial Hospital, Lodz, Poland
[21]Cancer Epidemiology Division, Cancer Council Victoria, Melbourne, Victoria, Australia
[22]Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, The University of Melbourne, Melbourne, Victoria, Australia
[23]Precision Medicine, School of Clinical Sciences at Monash Health, Monash University, Clayton, Victoria, Australia
[24]Department of Internal and Occupational Diseases, Hypertension and Clinical Oncology, Wroclaw Medical University, Wroclaw, Poland
[25]Department of Hematology, N. Copernicus Town Hospital, Torun, Poland
[26]Department of Hematology, Institute of Hematology and Transfusion Medicine, Warsaw, Poland
[27]Department of Haematology, Military Institute of Medicine, Warsaw, Poland
[28]Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal
[29]Molecular Oncology Research Center, Barretos, São Paulo, Brazil
[30]Department of Hematology, University Hospital of Salamanca, IBSAL, Salamanca, Spain
[31]Department of Hematology, Jagiellonian University Medical College, Cracow, Poland
[32]Department of Clinical Immunology, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark
[33]Department of Hematology, University Hospital of Cracow, Cracow, Poland
[34]Department of Experimental Hematooncolog, Medical University of Lublin, Lublin, Poland
[35]Department of Hematology, St. John's Cancer Center, Lublin, Poland
[36]Cancer Research Center of Lyon/Hospices Civils de Lyon, Lyon, France
[37]Department of Hematology, University Hospital Bydgoszcz, Bydgoszcz, Poland
[38]University of Utah, Salt Lake City, Utah
[39]Medical University of Lublin, Lublin, Poland
[40]Department of Hematology, Rydygier Specialistic Hospital, Cracow, Poland
[41]Department of Hematology, Odense University Hospital, Odense, Denmark
[42]Hematology Clinic, Holycross Cancer Center, Kielce, Poland
[43]Department of Pathology, University of Alabama at Birmingham, Birmingham, Alabama
[44]Genetic Epidemiology and Risk Assessment Program, Mayo Clinic Comprehensive Cancer Center, and Division of Biomedical Statistics and Informatics, Department of Health Sciences Research, Mayo Clinic, Rochester, Minnesota

Correspondence
Federico Canzian, Genomic Epidemiology Group, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany.
Email: f.canzian@dkfz.de

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Abstract

Gene expression profiling can be used for predicting survival in multiple myeloma (MM) and identifying patients who will benefit from particular types of therapy. Some germline single nucleotide polymorphisms (SNPs) act as expression quantitative trait loci (eQTLs) showing strong associations with gene expression levels. We performed an association study to test whether eQTLs of genes reported to be associated with prognosis of MM patients are directly associated with measures of adverse outcome. Using the genotype-tissue expression portal, we identified a total of 16 candidate genes with at least one eQTL SNP associated with their expression with $P < 10^{-7}$ either in EBV-transformed B-lymphocytes or whole blood. We genotyped the resulting 22 SNPs in 1327 MM cases from the International Multiple Myeloma

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rESEarch (IMMEnSE) consortium and examined their association with overall survival (OS) and progression-free survival (PFS), adjusting for age, sex, country of origin and disease stage. Three polymorphisms in two genes (*TBRG4*-rs1992292, *TBRG4*-rs2287535 and *ENTPD1*-rs2153913) showed associations with OS at $P < .05$, with the former two also associated with PFS. The associations of two polymorphisms in *TBRG4* with OS were replicated in 1277 MM cases from the International Lymphoma Epidemiology (InterLymph) Consortium. A meta-analysis of the data from IMMEnSE and InterLymph (2579 cases) showed that *TBRG4*-rs1992292 is associated with OS (hazard ratio = 1.14, 95% confidence interval 1.04-1.26, $P = .007$). In conclusion, we found biologically a plausible association between a SNP in *TBRG4* and OS of MM patients.

KEYWORDS
eQTL, genetic polymorphisms, multiple myeloma, overall survival, progression-free survival

1 | INTRODUCTION

Multiple myeloma (MM) is a malignancy of terminally differentiated plasma cells, which are primarily resident in the bone marrow. MM is the second most common hematological malignancy, with an annual crude incidence rate of 6.5 and 8 new cases per 100 000 inhabitants in Europe and in the United States of America, respectively.[1]

The advances in therapy made in the last decade have resulted in a considerable increase in patient survival. However, MM remains an incurable disease for most patients, who eventually relapse. The clinical course of MM is characterized by a high degree of heterogeneity, with long-term responders to therapy who survive long enough to eventually die of other causes, and patients who are refractory to any therapy and succumb very quickly to the disease.[2]

Gene expression profiling (GEP) is being widely used for tumor classification and prognosis and can effectively identify patients with very poor outcome. Numerous prognostic gene signatures have been identified in the past years; some of them were identified agnostically from direct comparison of patients with different survival while others were informed by genes relevant to the biology of MM.[3-11] Moreover, GEP has been able to classify patients based on their response to certain kinds of therapy, which could be valuable to personalize treatments given the vast heterogeneity of treatments and drug combinations.[12,13]

Over recent years, single nucleotide polymorphisms (SNPs) have been found associated with MM survival, through candidate[14-18] or genome wide association studies (GWAS).[19,20] However, the influence of germline variants on MM outcome remains a poorly explored field and few studies have identified SNPs associated with a different response to specific therapies.[21]

Recent evidence derived from large projects such as the genotype-tissue expression (GTEx) database have identified SNPs as expression quantitative trait loci (eQTLs), strongly associated with gene expression.[22] eQTLs have been successfully used as surrogates of direct measurement of gene expression to study disease etiology.[23,24] In most cases they are located in physical proximity to the genes whose expression they influence ("cis-eQTLs," usually mapping to promoter or enhancer regions), while some eQTLs are located in a different chromosomal region, or even on a different chromosome from the gene whose expression is affected ("trans-eQTLs"). Interestingly, it has been shown that polymorphisms associated with complex traits in GWAS, including risk of many cancers, are enriched in eQTLs.[24,25] Considering that the expression of several genes is associated with MM prognosis we hypothesized that SNPs that affect expression levels of those genes might also be associated with prognosis.

We performed an association study within the International Multiple Myeloma rESEarch (IMMEnSE) consortium to examine SNPs that act as eQTLs for genes included in expression signatures that have been previously shown to influence MM survival. We hypothesize that these eQTLs could be used as markers of outcome. We attempted to replicate the top associations in the International

What's new?

Multiple myeloma (MM) remains incurable for most patients, although recent therapeutic advances have extended survival. MM is highly heterogeneous, but gene expression profiling can identify patients with poor outcomes and classify patients by how they will respond to drugs. Here, the authors evaluate certain genetic loci that influence the amount of RNA transcript produced, called expression quantitative trait loci (eQTLs). They found two eQTLs of genes associated with MM prognosis that were directly associated with adverse outcomes. These results provide a proof-of-concept that eQTLs can serve as a surrogate for gene expression profile as a predictor of survival, and they are much easier to measure.

Lymphoma Epidemiology (InterLymph) consortium and performed a meta-analysis of results from both consortia.

2 | MATERIALS AND METHODS

2.1 | Study samples

2.1.1 | International Multiple Myeloma rESEarch (IMMEnSE) consortium

The first phase of the association study was performed in the IMMEnSE consortium, which has been described elsewhere.[26] Each collaborating institution retrospectively collected clinicopathological data from medical records on age, sex, country of origin, disease stage (Durie-Salmon and/or International Staging System [ISS]), and type of first-line therapy, response to first-line therapy, progression and vital status. We analyzed 1302 MM patients with staging information for the Durie-Salmon system and 1064 subjects with staging information for the ISS, while 1050 patients had data for both (Table 1). MM cases were diagnosed according to the IMWG criteria from 2001 to 2015 and 640 were treated with bortezomib/immunomodulatory drugs which we defined as "recent therapies."

2.1.2 | International Lymphoma Epidemiology (InterLymph) consortium

MM studies from InterLymph consisted of nine participating studies of European ancestry with genotype and phenotype information (2434 cases and 3446 controls), which was pooled to perform genome-wide association studies (GWAS) for risk and survival. In total, the primary InterLymph dataset had 885 cases with stage information (ISS). A secondary InterLymph survival dataset consisting of 392 patients diagnosed with MM with follow-up and disease stage available from The University of Texas/MD Anderson Cancer Center (MDACC) and University of California San Francisco in the United States was added.

Characteristics of study participants are summarized in Table 1.

2.2 | SNP selection

We selected a comprehensive list of genes whose expression levels were associated with poor MM prognosis in the literature.[3-5,8,9,27] We also identified GEP signatures associated with differential response to therapy.[13] From this review, we assembled a list of 283 genes and searched for eQTLs associated with the expression levels of those genes using the browser of the genotype-tissue expression project GTEx (http://www.gtexportal.org).[28] For our study, the cis window established from the browser was 1 megabase upstream and downstream of the transcriptional start site of each gene. We performed these queries using the expression data on the tissues represented in

TABLE 1 Study populations

		InterLymph	
	IMMEnSE	Primary	Secondary
Country of origin			
Italy	124		
Poland	793		
Spain	103		
Portugal	30		
Denmark	260		
Hungary	17		
USA		765	392
Canada		120	
Total	1327	885	392
Median age (25%-75% percentiles)	61 (54-67)	61 (54-68)	60 (53-67)
Gender			
Males	52%	63%	56%
Females	48%	37%	44%
Disease stage Durie-Salmon[a]			
1	186	71	–
2	320	83	–
3	808	419	–
Total	1316	574	–
Disease stage ISS[a]			
1	323	178	156
2	347	466	127
3	393	241	109
Total	1064	885	392
First line therapy[a,b]			
New	640	–	–
Old	687	–	–
Total	1327	–	–
Median overall survival months (25%-75% percentiles)	39 (20.5-69.47)	60 (31-93)	55 (28-81)
Median progression-free survival months (25%-75% percentiles)	23 (11.70-43.72)	NA	NA

[a]The sum does not add up to the total of subjects due to missing data.
[b]New therapies are those based on proteasome inhibitors and/or immunomodulating drugs; old therapies are all others.

GTEx that are closest to the cells of interest for MM, that is, EBV-transformed B-lymphocytes (from 114 samples) and whole blood (from 338 samples). We ranked the eQTLs according to *P*-values of association with gene expression. Ten of these genes (*RPS28*, *YWHAZ*, *CNDP2*, *TBRG4*, *HLA-DPA1*, *DHFR*, *RAB2A*, *SERPINB1*, *HLA-DRB1* and *IKZF1*) have significant eQTLs in both tissues while six other genes (*ACTR2*, *HELLS*, *ENTPD1*, *CCND2*, *CCND1* and *ANK3*) had eQTLs in at least one of the tissues analyzed. For each gene, we selected at least one eQTL while considering the linkage disequilibrium (LD) among eQTLs. The final list included 22 independent SNPs from these 16 genes (Table 2).

2.3 | Genotyping and quality control

2.3.1 | IMMEnSE

Genomic DNA was extracted from peripheral blood using the QIAampR 96 DNA QIAcubeR HT Kit and stored at −20°C till use. All the genotyping assays were carried out in 384-well format, with 10 ng of DNA from each subject using TaqMan (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.) or KASP (LGC Genomics, Berlin, Germany) SNP genotyping assays. For quality control about 5% of the samples were interspersed in the plates as duplicated. Samples with a call rate lower than 80% (N = 184) were discarded.

2.3.2 | InterLymph GWAS (primary and secondary)

Samples were genotyped using the Affymetrix 6.0 and Illumina (610 Quad, Human660W-quad Beadchip, Omni5, OmniExpress Beadchip, Oncoarray) platforms. Each of the GWAS was subjected to rigorous standardized quality control independently prior to imputation, which was performed via the Michigan imputation server (https://imputationserver.sph.umich.edu/) based on the Haplotype Reference Consortium (HRC).[29] After imputation, each site was filtered to include only imputed variants with information score >0.6 and further quality controls checks were implemented (genotype rate >95%, minor allele frequencies >0.01 and Hardy-Weinberg equilibrium [HWE] > $\times 10^{-5}$ in controls). Finally, the data were pooled and final quality control was performed on the pooled GWAS set including checks for missingness, duplicates, sex mismatch, abnormal heterozygosity, cryptic relatedness, population outliers (principal components analyses: Eigenstrat) and genomic inflation (λ > 1.00). Additional information on the MM GWAS studies contributing in the InterLymph consortium is showed in Supplementary Table 1.

TABLE 2 List of selected SNPs

Gene	Gene signature	SNP	Alleles major/minor	GTE × *P*-value[a]
RPS28	Kuiper 2012[11]	rs2972572	A/G	7.9×10^{-42}
DHFR		rs2560424	C/T	1.4×10^{-16}
		rs7387	T/A	1.4×10^{-16}
RAB2A		rs948421	T/C	1.2×10^{-10}
HLA-DQB1	Moreaux 2013[3]	rs1140347	T/C	2.3×10^{-30}
		rs1063355	T/C	2.1×10^{-29}
HLA-DRB1		rs66859861	C/T	1.7×10^{-18}
		rs9270917	G/T	4.7×10^{-29}
SERPINB1		rs62391542	C/T	8.6×10^{-08}
HLA-DPA1		rs116102562	T/C	9.3×10^{-16}
		rs1054026	G/C	4.2×10^{-15}
YWHAZ	Shaughnessy 2007[8]	rs3134353	A/T	4.5×10^{-18}
TBRG4		rs1992292	T/C	3.5×10^{-08}
		rs2289375	C/T	3.1×10^{-10}
CNDP2	Decaux 2008[4]	rs8084058	A/G	8.8×10^{-09}
		rs4891557	C/T	4.4×10^{-09}
ACTR2	Terragna 2016[13]	rs4671647	C/T	6.9×10^{-7}
HELLS		rs7100415	G/C	5.4×10^{-6}
ENTPD1		rs2153913	G/C	1.3×10^{-21}
CCND2		rs3217860	A/G	1.4×10^{-6}
CCND1		rs7102758	A/G	1.1×10^{-6}
ANK3		rs7072106	C/G	2.7×10^{-12}

[a]*P*-values of association between single nucleotide polymorphism (SNP) genotypes and level of expression of the respective gene. The data used for the analyses described in this manuscript were obtained from GTEx Analysis Release V7, accessed October 10, 2017.

2.4 | Statistical and bioinformatic analyses

Survival analysis in IMMEnSE was performed with Cox proportional hazards regression, calculating hazard ratios (HRs) and 95% confidence intervals (CIs), using overall survival (OS) and progression-free survival (PFS) as endpoints. OS was defined as the time interval between MM diagnosis and death or last follow-up. PFS was defined as the time interval between the ASCT (autologous stem cell transplantation) or high-dose treatment (for patients not eligible for ASCT) until documented progression or until the last progression-free examination. All analyses were adjusted for age at diagnosis, sex, country of origin, MM stage (calculated with the Durie-Salmon or ISS system) and type of first-line therapy, defined as treatment based on bortezomib/immunomodulatory drugs ("recent therapies") or any other regimen (such as vincristine/adriamycin/dexamethasone or melphalan/prednisone, "chemotherapy-based only therapies"). The statistical analysis was performed using per-allele and codominant models. We considered the threshold of statistical significance, using a Bonferroni correction, to be $P < .0023$ (0.05/22 SNPs). A stratified analysis by type of first line therapy was also performed for the six polymorphisms selected from the signature of Terragna et al.[13] In addition, we performed the same analysis adjusted by bone lesions for the two polymorphisms in *TBRG4*, since this gene is implicated in bone-related disease.[30]

The InterLymph survival GWAS data were analyzed using Gwasurvivr, an R package for genome-wide survival analysis[31] with Cox proportional hazard models adjusting for age, sex, site, 10 principal components from the GWAS and ISS stage.

Results from IMMEnSE and InterLymph (primary and secondary) GWAS were meta-analyzed according to a fixed effect model. The results of the single SNPs were not adjusted for type of first line therapy which was available only in IMMEnSE but not in InterLymph.

To identify the regulatory potential of selected SNPs and the regions nearby, we used HaploReg (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php)[32] and RegulomeDb (http://regulome.stanford.edu).[33]

3 | RESULTS

For IMMEnSE, the overall genotyping call rate was 92.3%, the minimum call rate observed was 91.9% (rs2972572) and the maximum 98.2% (rs1992292). The concordance between duplicates was of 99.9%. Five of the selected SNPs (*HLA-DQB1*-rs141471663, *HLA-*

TABLE 3 Results of the association between overall survival (OS) of multiple myeloma (MM) patients and expression quantitative trait loci (eQTLs) in the IMMEnSE population[a]

SNP	Alleles (M/m)[b]	N of patients	N of deceased patients	Allelic model		Codominant model			
				HR_{het} (95% CI)	*P*	HR_{het} (95% CI)	*P*	HR_{hom} (95% CI)	*P*
rs2972572	A/G	864	214	1.06 (0.87-1.28)	.564	1.16 (0.84-1.60)	.369	1.11 (0.75-1.63)	.604
rs1063355	T/G	836	206	1.06 (0.90-1.25)	.454	1.10 (0.78-1.55)	.563	1.13 (0.81-1.56)	.477
rs1140347	G/A	894	249	0.94 (0.82-1.07)	.376	1.01 (0.64-1.59)	.96	0.89 (0.68-1.16)	.394
rs66859861	C/T	890	240	0.98 (0.84-1.15)	.83	1.20 (0.84-1.73)	.311	1.00 (0.73-1.39)	.973
rs3134353	T/A	984	265	1.16 (0.97-1.39)	.103	1.22 (0.84-1.79)	.297	1.38 (0.93-2.04)	.106
rs8084058	G/A	970	262	0.97 (0.81-1.15)	.755	0.93 (0.66-1.32)	.695	0.93 (0.65-1.34)	.705
rs4891557	C/T	966	260	0.94 (0.76-1.17)	.586	0.87 (0.66-1.13)	.298	1.09 (0.62-1.92)	.743
rs1992292	T/C	984	265	1.23 (1.05-1.45)	**.012**	1.59 (1.18-2.15)	**.002**	1.52 (1.08-2.16)	**.017**
rs2289375	C/T	956	259	1.16 (0.96-1.38)	.106	1.33 (1.02-1.73)	**.034**	1.19 (0.79-1.81)	.408
rs1054026	G/C	984	263	0.87 (0.70-1.08)	.223	0.70 (0.37-1.33)	.275	0.64 (0.35-1.19)	.163
rs2560424	C/T	982	265	0.79 (0.64-0.96)	**.023**	0.81 (0.62-1.04)	.105	0.59 (0.34-1.03)	.065
rs116102562	T/C	935	243	0.97 (0.65-1.44)	.886	1.06 (0.33-3.41)	.922	0.99 (0.37-2.66)	.982
rs948421	T/C	978	265	1.17 (0.97-1.39)	.094	1.31 (0.90-1.90)	.152	1.42 (0.96-2.10)	.076
rs62391542	C/T	941	254	1.00 (0.85-1.17)	.984	1.33 (0.90-1.96)	.146	1.08 (0.76-1.52)	.657
rs7387	T/A	982	267	1.26 (1.03-1.54)	.027	1.37 (0.78-2.41)	.273	1.68 (0.97-2.91)	.065
rs4671647	C/T	1014	276	1.28 (0.73-1.06)	.18	0.87 (0.68-1.12)	.291	0.77 (0.50-1.18)	.239
rs7100415	G/C	981	272	0.99 (0.84-1.18)	.954	1.00 (0.77-1.31)	.961	0.98 (0.68-1.40)	.909
rs2153913	G/C	1022	277	**0.88 (0.75-1.05)**	**.146**	**0.71 (0.54-0.94)**	**.017**	0.79 (0.58-1.08)	.149
rs3217860	A/G	1012	272	0.87 (0.71-1.06)	.181	0.82 (0.63-1.05)	.13	0.90 (0.53-1.52)	.7
rs7102758	A/G	1018	277	0.95 (0.73-1.24)	.718	0.92 (0.70-1.22)	.589	1.02 (0.73-1.43)	.891
rs7072106	C/G	1017	272	1.05 (0.88-1.27)	.556	1.09 (0.85-1.40)	.484	1.07 (0.67-1.0)	.787

Note: P values < .05 are showed in bold.
[a]Adjusted for age, sex, country of origin, ISS disease stage and kind of first line therapy.
[b]M = major allele; m = minor allele.

DQB1-rs1130456, *HLA-DRB1*-rs66859861, *SERPINB1*-rs62392542 and *HLA-DPA1*-rs116102562) were not in HWE. All those SNPs were located in chromosome six where the MHC complex is located as well. It is well known that this particular genomic region is not neutral from the point of view of natural selection and is also known to contain duplicated sequences and copy number variants (CNVs),[34,35] and deviation from HWE may be expected.[34] Considering also that some of these SNPs (rs1140347 and rs62391542) are also not in HWE in the 1000 Genomes Project, and, on the other hand, that concordance of genotypes of duplicated samples in our study was 100% for these SNPs, we included them in further statistical analyses.

3.1 | Discovery phase (IMMEnSE results)

The most significant association was seen for *TBRG4*-rs1992292 which showed an association with OS when adjusted for ISS disease stage system (Table 3). The C/T genotype is associated with a worse OS in our set of patients (HR = 1.59, 95% CI = 1.18-2.15, *P* = .0024) in the codominant model of inheritance. Additionally, rs2289375, another independent SNP in the same gene, showed weaker evidence of association in the same direction of *TBRG4*-rs1992292 (HR = 1.33, 95% CI = 1.06-1.67, *P* = .013). These two SNPs were also nominally associated with a worse PFS (Table 4). Results were similar regardless of the staging system used for adjustment (Durie-Salmon or ISS).

Additionally, we found several associations with different endpoints at the nominal level of $P < .05$. Namely, the *ENTPD1*-rs2153913 SNP showed associations with OS when considering all cases (HR = 0.71, 95% CI = 0.54-0.94, *P* = .017, for the heterozygotes in the codominant model) and cases treated with new therapies (HR = 0.61, 95% CI = 0.37-0.98, *P* = .043), but not cases treated with the old therapies (HR = 0.78, 95% CI = 0.55-1.11, *P* = .168).

Both polymorphisms in *TBRG4* showed associations with OS when adjusting by bone lesions. In particular, the strongest association was observed for *TBRG4*-rs1992292 for the codominant model of inheritance (HR_{het} = 2.21, 95% CI = 1.49-3.28, *P* = .0001). All results for these analyses are reported in Supplementary Table 2.

All the results presented and Tables 3 and 4 were adjusted for ISS, while the results adjusted using Durie-Salmon staging are showed in Supplementary Tables 3 and 4.

TABLE 4 Results of the association between progression-free survival (PFS) of multiple myeloma (MM) patients and expression quantitative trait loci (eQTLs) in the IMMEnSE population[a]

						Codominant model				
Gene	SNP	Alleles (M/m)[b]	N of patients	N of deceased patients	Allelic model HR_{het} (95% CI)	*P*	HR_{het} (95% CI)	*P*	HR_{hom} (95% CI)	*P*
RPS28	rs2972572	A/G	1072	298	1.03 (0.88-1.20)	.703	1.11 (0.85-1.48)	.429	1.06 (0.77-1.46)	.716
HLA-DQB1	rs1063355	T/G	1043	293	1.05 (0.92-1.21)	.468	1.09 (0.82-1.44)	.559	1.10 (0.83-1.45)	.493
HLA-DQB1	rs1140347	G/A	1098	332	0.96 (0.85-1.08)	.474	1.07 (0.74-1.54)	.726	0.92 (0.73-1.17)	.515
HLA-DRB1	rs66859861	C/T	1083	323	0.95 (0.83-1.08)	.430	0.95 (0.70-1.29)	.757	0.90 (0.68-1.18)	.44
YWHAZ	rs3134353	T/A	1201	355	1.05 (0.90-1.22)	.512	1.08 (0.80-1.47)	.602	1.12 (0.81-1.54)	.491
CNDP2	rs8084058	G/A	1179	345	0.99 (0.85-1.16)	.946	0.95 (0.70-1.27)	.718	0.97 (0.71-1.33)	.87
CNDP3	rs4891557	C/T	1174	348	0.91 (0.76-1.10)	.336	0.85 (0.68-1.07)	.184	0.98 (0.61-1.60)	.96
TBRG4	rs1992292	T/C	1196	350	1.18 (1.03-1.37)	**.018**	1.49 (1.15-1.93)	**.002**	1.40 (1.04-1.90)	**.027**
TBRG4	rs2289375	C/T	1165	345	1.17 (1.00-1.37)	**.044**	1.33 (1.06–1.67)	**.013**	1.23 (0.85-1.76)	.269
HLA-DPA1	rs1054026	G/C	1200	352	0.99 (0.81-1.20)	.923	0.89 (0.49-1.60)	.699	0.91 (0.52-1.59)	.732
DHFR	rs2560424	C/T	1202	356	0.85 (0.71-1.01)	.069	0.87 (0.69-1.09)	.225	0.68 (0.42-1.10)	.117
HLA-DPA1	rs116102562	T/C	1126	312	1.03 (0.72-1.47)	.872	1.14 (0.40-3.28)	.801	1.12 (0.46-2.72)	.802
RAB2A	rs948421	T/C	1192	351	1.10 (0.94-1.28)	.243	1.20 (0.87-1.66)	.253	1.25 (0.89-1.74)	.194
SERPINB1	rs62391542	C/T	1155	339	1.02 (0.89-1.17)	.773	1.34 (0.97-1.86)	.076	1.11 (0.83-1.49)	.468
DHFR	rs7387	T/A	1201	356	1.07 (0.91-1.27)	.411	1.13 (1.13-1.76)	.592	1.19 (0.77-1.84)	.431
ACTR2	rs4671647	C/T	1223	359	0.92 (0.78-1.08)	.319	0.85 (0.68-1.06)	.153	0.92 (0.65-1.32)	.67
HELLS	rs7100415	G/C	1185	351	0.98 (0.84-1.15)	.871	0.96 (0.76-1.20)	.702	0.97 (0.71-1.33)	.851
ENTPD1	rs2153913	G/C	1227	356	0.91 (0.78-1.06)	.219	0.80 (0.63-1.02)	.071	0.85 (0.63-1.13)	.261
CCND2	rs3217860	A/G	1223	355	0.89 (0.75-1.06)	.202	0.89 (0.71-1.11)	.286	0.80 (0.51-1.27)	.35
CCND1	rs7102758	A/G	1018	277	1.04 (0.83-1.30)	.736	1.06 (0.83-1.35)	.661	1.02 (0.76-1.37)	.907
ANK3	rs7072106	C/G	1017	272	1.07 (0.91-1.26)	.39	1.13 (0.91-1.41)	.261	1.10 (0.73-1.64)	.648

[a]Adjusted for age, sex, country of origin, ISS disease stage and kind of first line therapy. *P* values <.05 are showed in bold.
[b]M = major allele; m = minor allele.

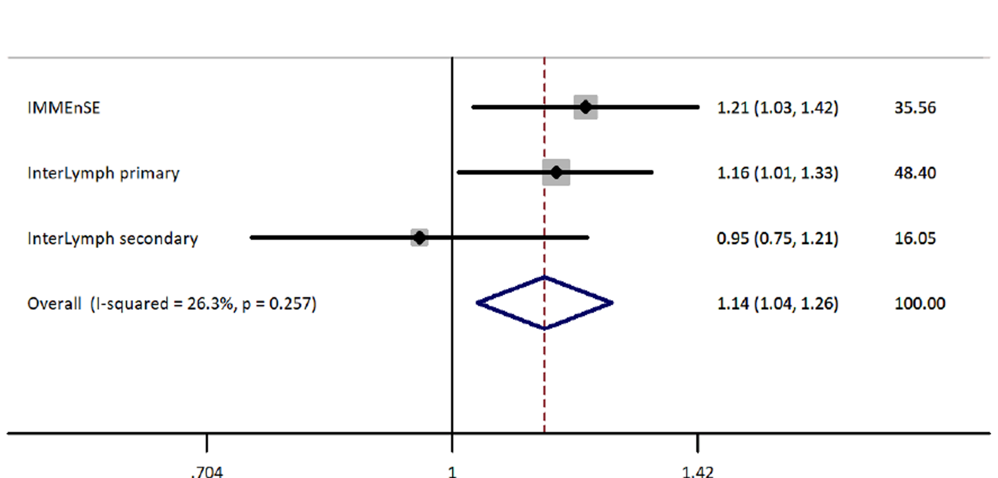

FIGURE 1 Forest plot for *TBRG4*-rs1992292 [Color figure can be viewed at wileyonlinelibrary.com]

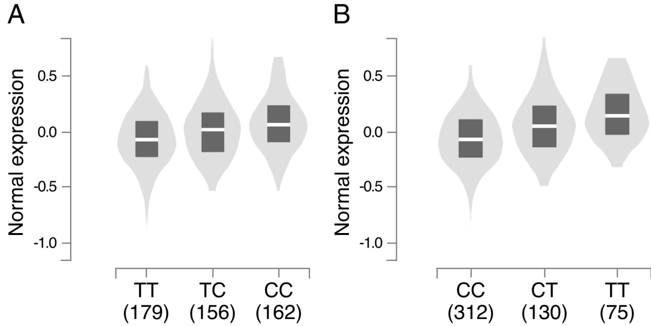

FIGURE 2 A, box plot of the association between rs1992292 and TBRG4 expression; B, box plot of the association between rs2289375 and TBRG4 expression. The data used for the analyses described in this manuscript were obtained from: GTEx Analysis Release V8, acccessed on 10/10/2019

3.2 | Replication phase (InterLymph)

Survival analysis in the InterLymph datasets was performed on the top three associations seen in IMMEnSE ($P < .05$): *ENTPD1*-rs2153913, *TBRG4*-rs1992292 and *TBRG4*-rs2289375. Associations with both polymorphisms in *TBRG4* replicated in the primary InterLymph dataset with OS: rs1992292 showed an HR = 1.16, 95% CI = 1.01-1.33, $P = .046$ and rs2289375 an HR = 1.24, 95% CI = 1.06-1.47, $P = .008$, considering the allelic model. The association with *ENTPD1*-rs2153913 was not replicated in this set (HR = 1.03, 95% CI = 0.88-1.19, $P = .731$). None of the above mentioned associations replicated in the additional set of cases from the secondary InterLymph dataset.

A total of 2579 cases were used for the meta-analysis and the polymorphism *TBRG4*-rs1992292 showed to be significantly associated with OS, with no heterogeneity between the three groups (HR = 1.14 95% CI 1.04-1.26, $P = .007$) and a forest plot for this analysis is shown in Figure 1. No evidence of association was observed for *ENTPD1*-rs2153913 (HR = 0.93, 95% CI 0.84-1.04, $P = .211$).

Kaplan-Meier curves for the survival of MM patients according to genotype at the two *TBRG4* SNPs are shown separately for IMMEnSE and the primary InterLymph dataset (Supplementary Figures 1 and 2).

4 | DISCUSSION

The investigation of germline variants that act as eQTL for genes whose expression is known to affect MM prognosis could be used to identify predictors of patient outcome. Based on this rationale, we tested whether eQTLs of genes included in expression signatures that define MM patients with poor prognosis are associated with adverse outcome and therefore could be used as genetic markers of prognosis.

Our results suggest that the minor alleles of the *TBRG4*-rs1992292 and *TBRG4*-rs2289375 SNPs are associated with a worse survival. *TBRG4* encodes for a regulator of transforming growth factor beta (TGF-β), which is involved in various cellular pathways, including the regulation of hematopoiesis, an important process for myeloma cell proliferation and survival.[36] Increased levels of TGF-β in the bone marrow microenvironment induce an increase of IL-6 and VEGF secretion, major cytokines involved in cancer cell proliferation and angiogenesis.[37] Moreover, it has been reported that downregulation of *TBRG4* contributes to arrest of cell cycle in the G1 phase, which ultimately leads to a better outcome in MM.[38] This gene was selected initially because it was reported that its higher expression is associated with a shorter survival in MM patients.[8]

TBRG4-rs1992292 is in strong LD ($r^2 = 0.935$ in European population [CEU] of the 1000 Genomes project) with rs6967730, that has a rank of 1f in RegulomeDB, indicating that it is likely to affect the binding of additional transcription factors and it is linked to expression of

TBRG4. In this regard, it has been reported that rs6967730 is located within a transcription factor binding site for CTCF (CCCTC-binding factor), a highly conserved zinc finger protein. CTCF can function as a transcriptional activator, a repressor or an insulator protein, blocking the communication between enhancers and promoters.[39] Therefore, rs6967730 could be responsible for changing the expression of *TBRG4* by modifying the binding site of CTCF. Even though the information provided by our and other studies is relevant, in-depth analysis of the biological role of the *TBRG4*-rs1992292 SNP in modulating MM survival, including mechanistic insights, is still needed.

According to our results, *TBRG4*-rs2289375 is associated with a worse survival of MM patients. This SNP has a RegulomeDB rank of 2b and is in LD (r^2 = 1) with rs3757573, which has a rank of 1f indicating that it could have a strong functional role in affecting the expression of *TBRG4*. The GTEx portal reports that the TT genotype is associated with a higher expression of *TBRG4* in both the tissues we considered, in line with our results where carriers of this genotype have a worse survival (Figure 2).

Finally, although *ENTPD1*-rs215391 did not replicate in the InterLymph datasets, this SNP, according to our bioinformatics analysis, has a clear biological link with MM. Indeed, GTEx reports that the C allele of *ENTPD1*-rs215391 decreases the expression of the *ENTPD1* gene, which translates into a lower production of adenosine which, in turn, results in a less active adenosine-mediated immunosuppressive pathway increasing the anticancer monitoring immune system. Considering the above, we cannot exclude a potential contribution of *ENTPD1*-rs215391 in MM outcome.

The study has some weaknesses: data on PFS and type of first line treatment were not available for InterLymph cases; therefore, we could not confirm the result obtained with PFS as endpoint. Another limitation is the lack of karyotype data which are involved in the heterogeneity observed in patient prognosis. Moreover, the selection of the eQTLs was limited to one or two eQTLs for each region that we selected (the ones showing associations with gene expression levels with the lowest *P*-values in GTEx) and therefore we could not exclude the possibility of having missed additional associations. Our results, however, represent a proof of principle that eQTLs could be used as MM survival markers and offer a starting point to further investigate in this direction alongside other known prognostic markers.

Standard eQTL analysis, which involves a direct association test between markers of genetic variation with gene expression levels, has many advantages. The main one is that the genotypes are not influenced by sample manipulation or by environmental variables since invariable throughout life of an individual. eQTL analysis can be performed in silico using available GWAS dataset and free bioinformatic tools as GTEx, which makes this kind of analysis basically costless compared to GEP which involves the use of expensive equipment and reagents.

The main strengths of the study are that our results were confirmed in two of three independent datasets with a large overall sample size with information on OS and stage.

In conclusion, we found biologically plausible associations between SNPs in *TBRG4* and OS of MM patients that should be investigated more deeply. eQTLs are a valid surrogate for GEP and are much easier to measure than GEP itself.

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CONFLICT OF INTEREST

The authors declared no potential conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ETHICS STATEMENT

The IMMEnSE study protocol was approved by the Ethics Committee of the Medical Faculty of the University of Heidelberg (reference number: S-004/2020). Following the guidelines of the Declaration of Helsinki, written informed consent was obtained from each participant.

ORCID

Angelica Macauda https://orcid.org/0000-0001-9820-5079
Juan Sainz https://orcid.org/0000-0002-9355-2423
Michelle A. T. Hildebrandt https://orcid.org/0000-0001-6769-6872
Federico Canzian https://orcid.org/0000-0002-4261-4583
Daniele Campa https://orcid.org/0000-0003-3220-9944

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SUPPORTING INFORMATION

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

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