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Review

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Genomics of cutaneous melanoma: focus on next-generation sequencing approaches and bioinformatics

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Abstract

Cutaneous melanoma is caused by the uncontrolled growth of epidermal melanocytes. Melanoma continues to be a rare form of skin cancer but causes the majority of skin cancer related deaths. For many years the scientific community has focused on the investigation of the pathogenesis leading to melanoma, with the aim of better understanding its complexity and the potential advancement of therapeutic strategies. In this paper, the genomic features characterising the development of cutaneous melanoma are reviewed. Next-generation sequencing technologies and bioinformatics tools are currently state-of-the-art approaches in basic, applied and clinical cancer research. In this review, most of the available tools for revealing the mutational landscape are outlined.

Keywords: Melanoma, bioinformatics, somatic mutations, next-generation sequencing

INTRODUCTION

Melanoma is a malignant tumour originating from melanocytes, the cells specialised to produce the melanin pigment. Melanocytes derive from the neural crest, a transient embryonic structure, consisting of highly migratory pluripotent cells, which give rise to a number of different cell types[1]. During development, melanocyte progenitors migrate, differentiate and colonize the skin-epidermis and hair follicles, the uvea

of the eye and mucous membranes throughout the body. Accordingly, melanoma can arise at all these sites, leading to phenotypically, histologically, clinically and genetically diverse types of disease. In Caucasian populations, the most common type of melanoma is cutaneous melanoma (CM), originating from the epidermal melanocytes of non-glabrous skin. Among skin cancers, melanoma is the most aggressive, and although it accounts for less than 5% of skin cancer incidence, it is responsible for the majority of related deaths[2]. In this review we will focus on CM, as there are many differences in the genetic background implicated in different types of melanoma, such as mucosal or uveal melanoma. A distinct melanoma subtype, often referred to as a subtype of CM, is acral melanoma, occurring on glabrous (nonhair-bearing) acral skin of palms, soles and nail beds, which will not be further discussed in this review.

During the last decades, a continuous increase of CM frequency rates has been observed in Caucasian populations worldwide, making CM the cancer with the most rapidly increasing occurrence. CM incidence varies significantly between populations from different geographic regions, with Australia and New Zealand presenting the highest incidence rates worldwide. In Europe, rates are lower, but still have shown a three-fold to five-fold increase during this time period[3]. CM occurrence differs substantially between European countries, with Switzerland showing the highest rate and Greece belonging to the group of low-incidence countries[4,5].

CM development is a complex multi-factorial process, arising through multiple etiologic pathways and involving the interplay of genetic and environmental risk factors. Among them, the most well-established risk factors are exposure to ultraviolet (UV) radiation, family history, and phenotypic traits carrying a strong genetic component - including hair and eye colour, and the number of common and atypical melanocytic nevi[2].

In this review, we will summarize the progress towards the genomic characterization of CM, recent advances achieved through the exploitation of next-generation sequencing (NGS) technologies, as well as the bioinformatics tools developed for the analysis of sequencing data.

GERM-LINE SUSCEPTIBILITY

Regarding the genetic background predisposing to melanoma, several susceptibility loci acting as high, moderate or low penetration genes, have been identified. Cyclin-dependent kinase inhibitor 2A (*CDKN2A*), the first familial melanoma gene identified[6,7], is found mutated in approximately 40% of melanoma high-density families. *CDKN2A* encodes for two distinct proteins, p16INK4A (p16) and p14ARF (p14), both involved in the regulation of the cell cycle[8]. The p16 and p14 mRNAs are transcribed from alternative first exons, so the related proteins have no similarity in their amino acid sequence, since they are translated in alternative reading frames. Mutations in p16 are predominantly loss of-function missense mutations, distributed throughout the protein, while in p14 inactivating mutations like whole gene deletions, insertions or splice-site mutations are mainly observed. Germ-line mutations in *CDK4* are much less frequent and were initially identified by screening for p16 interacting partners. A mutational hotspot in codon 24, leading to an arginine substitution, abrogates the capacity of p16 to inactivate the kinase, thus promoting the G1-S phase transition. Other mutations have been identified in genes of more moderate penetrance, including *BAP1*, *TERT*, *POT1*, *ACD*, *TERF2IP* and *MITF*[9]. Genome-wide association studies have also revealed numerous recurring single nucleotide polymorphisms (SNPs) associated with melanoma risk[10-13].

TOWARDS THE GENOMIC CHARACTERISATION OF MELANOMA

Identifying somatic mutations in the genome of melanoma is of great importance in order to understand the molecular basis of the disease's genesis and progression. A number of oncogenes and tumour suppressor genes have been found to carry causative mutations. The first oncogene identified in melanoma was *NRAS*[14], which is also found mutated in other cancers. In 2002 the *BRAF* V600E somatic mutation was identified[15] and is the most frequent mutation found in CM patients. Since then, the advances in sequencing

technology have enabled the application of massively parallel sequencing, thus dramatically changing our understanding of the somatic mutation landscape of melanoma. The first catalogue of somatic mutations of a cancer genome, at the whole-genome level concerned a melanoma cell line[16], indicated the presence of a great number of mutations per Mb and suggested a mutational signature related to UV exposure. Whole-exome sequencing studies exploiting clinical samples demonstrated that *NF1, ARID2, PPP6C, RAC1, SNX31, TACC1,* and *STK19* are genes significantly mutated in melanoma[17,18]. The Cancer Genome Atlas Skin Cutaneous Melanoma (SKCM-TCGA) project confirmed, through exome sequencing, previously reported melanoma oncogenes and tumour suppressors (*BRAF, NRAS, CDKN2A, TP53,* and *PTEN*) and identified several additional significantly mutated melanoma genes, namely, *MAP2K1, IDH1, RB1,* and *DDX3X*[19]. The study proposed the classification of CM into four major genomic subtypes, related to the presence of specific mutations in established driver genes. In particular, the proposed genetic subtypes are the *BRAF* mutant, *RAS* mutant, *NF1* mutant, and the triple wild-type. Low-frequency mutations were identified in the triple wild-type subtype in *KIT*, *CTNNB1*, *GNA11*, and *GNAQ*. More recently, the first large-scale study exploiting whole-genome sequencing supported the involvement of the non-coding genome in melanoma pathogenesis and revealed diverse carcinogenic processes across the different melanoma subtypes. Figure 1 summarizes the research on melanoma during the last decades, pinpointing key milestones in understanding its complexity.

MUTATION BURDEN AND SPECIFIC SIGNATURES IN MELANOMA

Sequencing of different cancers has revealed that the melanoma genome shows a substantial prevalence of somatic mutations[16,20,21]. Particularly, in CM an increased abundance of cytidine to thymidine (C > T) transitions is observed. This specific alteration is considered characteristic of a UV-light-induced mutational signature. A recent study, exploiting whole-genome sequencing of cutaneous, acral and mucosal melanomas, revealed distinct mutation profiles among these melanoma subtypes. The number of base substitutions and short insertions and/or deletions in CM was generally much higher than of those observed in acral and mucosal melanomas. In addition, the UV-related C > T transition was not observed in the latter melanoma subtypes. In contrast, somatic structural rearrangements were more frequent in acral and mucosal subtype[22]. These data suggest that different etiologic pathways are involved in the manifestation of diverse melanoma subtypes.

NEXT-GENERATION SEQUENCING APPROACHES AND BIOINFORMATICS

The significant progress towards the characterisation of the somatic mutational landscape of melanoma, can be mainly attributed to the rapid evolution of sequencing technologies during the last fifteen years. Nowadays, NGS has become the state-of-the-art tool in cancer research and is the most common and advanced technology for *de novo* somatic mutation detection. NGS technologies are in continuous development and improvement, both at the level of the applied protocols for library preparation and sequencing chemistry, but also at the bioinformatics level. A large number of bioinformatics tools have been developed for general pre-processing and basic analysis of NGS (WES/WGS) data with the aim of revealing altered variants for the cases under investigation. In this part of our review, we will focus only on tools developed for somatic mutation calling, bypassing those needed to reach this step of the analysis. Furthermore, we will discuss most of the available tools for driver-mutation identification, including the approaches that are used to achieve this step. Discriminating driver from passenger mutation remains a challenge from the experimental as well as the bioinformatics points of view[17,23-26]. In the case of melanoma, which is one of the cancers with the highest mutation burdens and heterogeneity, this problem is even more difficult to address, due to the confounding impact of melanoma's high mutation rate. The aim of this review is not to perform a comparison of the tools since more detailed evaluations are available[26-28].

The basic approach for somatic variance identification is to compare paired samples, i.e., analyse matched tumour-normal samples collected from the same patient. Most callers are structured after this notion and

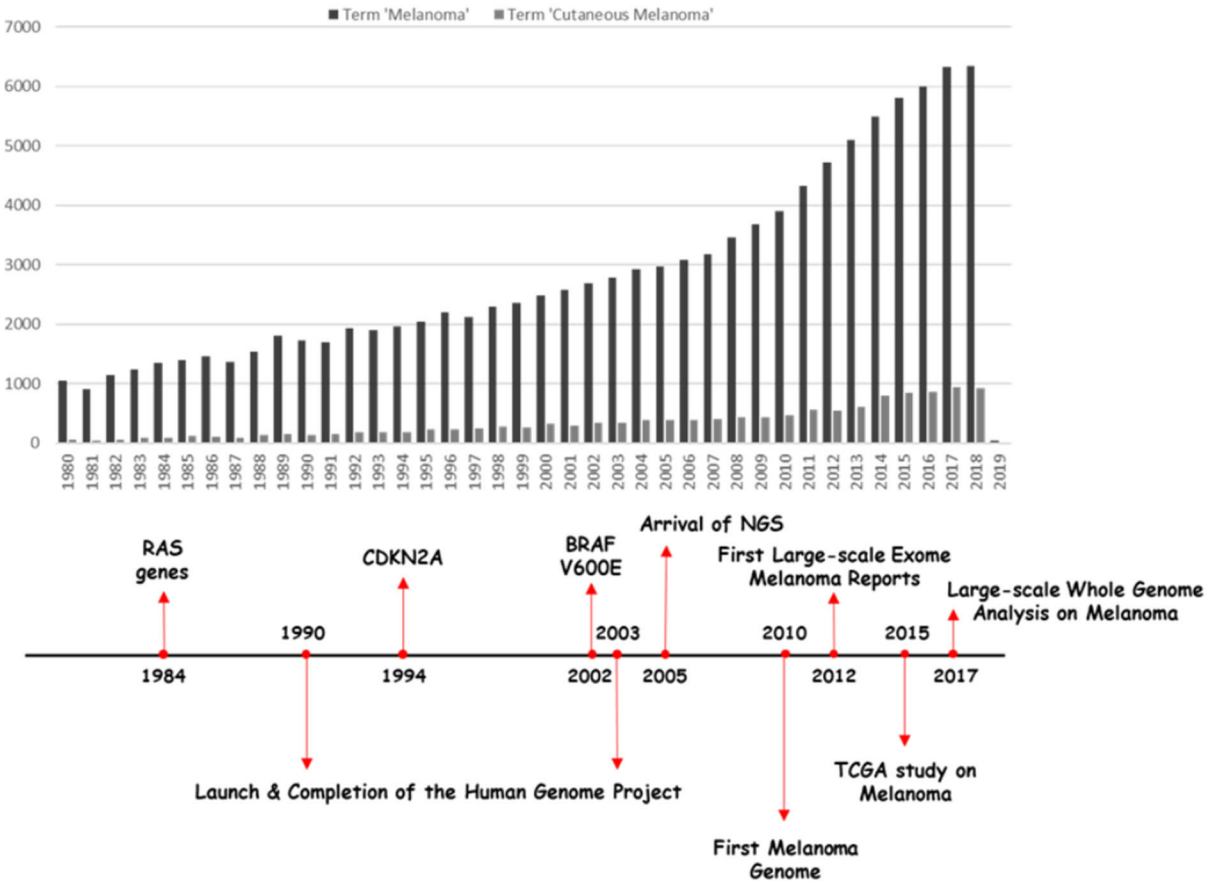

Figure 1. The number of publications per year on Pubmed (until 4th December 2018) using terms "melanoma" and "cutaneous melanoma" (upper), major landmarks concerning the study of melanoma (lower). NGS: next generation sequencing

use different approaches to extract the desired list of variants, meeting certain criteria. Among the strategies utilized are heuristic approaches combined with statistical tests, analysis and evaluation of a joint genotype likelihood, allele frequency or haplotype-based analyses, or exploitation of machine learning methods for variant classification. Apart from these, there are specialized tools that offer single-sample somatic mutation calling (lack of normal samples), through association with databases like COSMIC[29,30] and application of machine learning and statistical algorithms. Table 1 lists most somatic mutation callers based on their aforementioned strategic approaches.

As a latter step, after obtaining a list of somatic mutations, it is important to distinguish the driver mutations which actively contribute to carcinogenesis[79]. This can be accomplished through mutation frequency analysis, functional impact investigation or machine learning algorithms based on known sets of driver/passenger genes. Another approach followed is enrichment analysis on known pathways or networks. Table 2 summarizes several tools which focus on driver mutation identification, classified by the strategic approach used. It is important to mention that distinction of driver/passenger genes faces many challenges mostly due to lack of annotation, additive effects of passenger mutations or a possible change in roles during cancer progression and the development of tumour heterogeneity[28]. In a recent publication, our group presented a methodology combining functional impact analysis with pathway enrichment, to deal with a limited dataset, in order to distinguish important genes and possible drivers exploiting exome sequencing data from melanoma patients in Greece[80].

GENES BEARING CAUSATIVE SOMATIC MUTATIONS IN MELANOMA

One of the most well-established pathways commonly affected in melanoma is the mitogen activating protein kinase (MAPK) signaling cascade, governing cell growth and survival. *BRAF, NRAS* and *NF1* are the

Table 1. Somatic variance calling tools

Analysis tactic	Variant callers
Heuristic approaches	qSNP[31], RADIA[32], Shimmer[33], SOAPsnv[34], VarDict[35], VarScan2[36]
Joint genotype analysis	CaVEMan[37], FaSD-somatic[38], JointSNVMix2[39], SAMtools[40], Seurat*[41], SNVSniffer[42], SomaticSniper[43], Virmid[44]
Allele frequency	deepSNV[45], EBCall[46], LoFreq[47], LoLoPicker[48], MuTect[49], Strelka[50]
Haplotype analysis	FreeBayes[51], HapMuC[52], LocHap[53], MuTect2[49], Platypus[54]
Machine learning	BAYSIC[55], MutationSeq[56], SNooPer[57], SomaticSeq[58]
Single-sample analysis	GATKcan[59], ISOWN[60], OutLyzer[61], Pisces[62], SiNVICT[63], SomVarIUS[64]
Structural or copy number variation calling	APOLOH[65], BIC-Seq[66], BreakDancer[67], Break-Pointer[68], CNVkit[69], CoNIFER[70], Delly[71], HYDRA[72], GASV[73], GASVPro[74], Meerkat[65], PeSV-Fisher[75], VariationHunter-CommonLaw[76]
RNA-seq variant calling	eSNVdetect[77], SNPiR[78], VarDict[35], VarScan2[36]

Table 2. Driver mutation calling tools

Analysis tactic	Driver callers
Functional impact	CanPredict[81], Condel[82], FATHMM[83], GERP++[84], GOSS[85], MutationAssessor[86,87], MutationTaster[88], Oncodrive-fm[89], PMUT[90], PolyPhen-2[91], PROVEAN[92], SIFT[93], SNPs3D[94], TransFIC[95]
Mutation frequency	DrGaP[96], MuSiC[97], MutSig/MutSigCV[25], Youn and Simon[98]
Machine learning	CHASM[99,100], DMI[101]
Structural or copy number focus	ADMIRE[102], CMDS[103], GISTIC2[104], JISTIC[105]
Positional/structural clustering	iPAC[106], NMC[107]
Pathway/network analysis	BioInfoMiner[108], Dendrix[109], GSEA[110], HotNet[109], MEMo[111], Multi-Dendrix[112], NetBox[113], PathScan[114], Patient-oriented gene sets[115], RME[116]

most frequently mutated genes of this pathway. Other pathways found significantly altered in CM include the phosphoinositide 3-kinase (PI3K) pathway, tumour protein 53 (TP53) signaling, cell cycle regulation and the telomere length maintenance pathway. In the next section, the most significant genes involved in such key processes, harboring driver mutations, are summarized.

BRAF

The *BRAF* gene encodes a serine/threonine protein kinase, belonging to the RAF family. This protein acts as a downstream effector of *RAS*-signalling in the MAPK cascade, affecting cell proliferation and survival. Mutations in this gene have been identified in various cancers. According to the COSMIC database 44% of melanomas arising from skin tissue have mutations in *BRAF*. In non-acral CM, the *BRAF* mutation of the kinase-activation domain at amino acid position 600, is the most common somatic mutation. Interestingly, *BRAF* V600E mutation results from a T→A transversion and not a C>T substitution, which is characteristic of UV light induced mutagenesis. Nevertheless, epidemiological and genomic evidence implies that UV radiation contributes to the formation of *BRAF* V600E. Soon after the characterization of *BRAF* V600E mutation in melanomas, it became apparent that its distribution greatly differs among different melanoma subtypes[117]. In particular, *BRAF* V600E mutations are more common in younger CM patients, whose melanomas arise on intermittently sun-exposed skin, on anatomical sites such as the trunk and proximal extremities. In contrast, melanomas arising on chronically sun-damaged skin, usually on anatomical sites like head, neck and the distal extremities of older individuals, have infrequent *BRAF* mutations, with *BRAF* V600K being more frequent than BRAF V600E[118]. Acral melanomas bear *BRAF* mutations much less frequently. Targeting the *BRAF*-V600E mutant protein with specific inhibitors exposed new therapeutic aspects for the management of such an aggressive disease. The oncogenic activation of BRAF mutations is considered a necessary but not sufficient condition to transform melanocytes to melanoma cells, a suggestion which is also supported by the frequent occurrence of such mutations in benign nevi[119].

RAS

RAS proteins are small GTPases functioning as GDP-GTP-regulated binary switches that control many fundamental cellular processes. RAS proteins connect a great variety of upstream signals from activated

membrane receptors to downstream pathways controlling the cell cycle, growth, apoptosis, and senescence[119]. The *HRAS, KRAS,* and *NRAS* oncogenes were the first human oncogenes to be discovered[120]. In the case of CM, NRAS mutations are found in 17% of the cases, according to COSMIC database[29]. NRAS hot-spot mutations are mutually exclusive of BRAF hot-spot mutations. *HRAS* and *KRAS* mutations are much less frequent in CM. Regarding *NRAS*, the most common mutations cause a change of the amino acid at position 61, lying at the GTP-binding domain. These substitutions disrupt the GTPase activity of the protein, locking it in its active conformation[121].

NF1

NF1 is a tumour suppressor gene encoding for a direct negative regulator of RAS signaling[122]. In particular, NF1 is a GTPase-activating protein known to downregulate RAS activity by stimulating the hydrolysis of GTP and returning the protein to its inactive form. A significant enrichment of NF1 mutations was found in BRAF and NRAS wild-type melanomas[17,18]. In the TCGA study NF1 was found as the third most frequently observed significantly mutated gene of the MAPK pathway[123]. Mutations in the NF1 gene are loss of function mutations, mainly nonsense point mutations[124], which can be considered as an alternative way to activate the MAPK signaling pathway.

TERT

The *TERT* gene encodes for the telomerase reverse transcriptase, the catalytic subunit of the telomerase ribonucleoprotein, essential for the maintenance of telomeres and chromosomal stability. Recurrent somatic mutations in the *TERT* promoter have been characterised in CM, with high frequency in sporadic melanoma. Specifically, the two hot-spot mutations, located at -124 and -146bp relative to the transcriptional start site, are C>T transitions, consistent with a UV signature mutational profile[125,126]. In a recent study exploiting whole genome sequencing, 86% of CM cases were found mutated at one or more out of four positions upstream of the transcriptional start site[22]. All these mutations are mutually exclusive and create new binding sites for the E26 transformation-specific family transcription factor GA-binding protein. Recent evidence suggests that *TERT* promoter mutations result in *TERT* over expression[127,128]. They are established after MAPK-pathway activating mutations, but still during the early stage of melanoma progression[129].

CDKN2A

CDKN2A is a well characterised tumour-suppressor gene, found to harbour somatic alterations in a wide variety of different tumour types[128]. Regarding CM, in addition to its association with familial melanoma, somatic alterations resulting in *CDKN2A* inactivation are also frequently observed in sporadic melanoma. The most frequent alteration is the deletion of the *CDKN2A* gene, reported in 41% of CM[129]. *CDKN2A* expression is additionally regulated at the epigenetic level, mainly by methylation of its promoter and subsequent gene silencing. The two proteins encoded by *CDKN2A*, p16 and p14, have distinct roles in the regulation of the cell cycle. p16 modulates G1 to S phase transition by inhibiting the kinase activity of cyclin dependent kinases 4 and 6 (CDK4 and CDK6), while p14 acts through TP53 stabilisation. Biallelic loss of *CDKN2A* and subsequent disruption of the G1/S checkpoint, is believed to be a crucial step in melanoma progression towards transition to the invasive phenotype[127].

TP53

TP53 is a well-known tumour suppressor gene, involved in the transcriptional regulation of several target genes. *TP53* is mutated in 27 different types of cancer[130]. Regarding melanoma, 15% of cases harbour mutations in *TP53*[29]. Based on mutational studies, comparing primary melanomas and metastases, *TP53* was found to be more frequently mutated in melanoma metastases, indicating that *TP53* mutations may arise later during melanoma progression[118].

PTEN

PTEN is a tumour-suppressor gene, coding for the phosphatidyl-inositol-3,4,5-triphosphate 3-phosphatase.

PTEN phosphatase is a fundamental regulator of the PI3K/AKT pathway, exerting its inhibitory effects on AKT signaling, by dephosphorylating PIP3. PIP3 acts as a second messenger, triggering a number of signaling cascades- among them AKT- which play a key role in processes like cell survival and proliferation, apoptosis, and cellular metabolism[131]. Somatic mutations in *PTEN*, primarily deletions but also loss-of-function SNVs - 18 % and 8% respectively in CM[129], result in PTEN inactivation and promote cell survival through sustained activation of the PI3K signaling pathway[132].

MITF

Microphthalmia-associated transcription factor (MITF) is a basic helix-loop-helix/leucine zipper transcription factor required for melanocyte development. MITF is essential for establishing the melanocytic lineage during differentiation of neural crest cells[133]. Transcriptional targets of MITF, include genes encoding for components of melanosomes, enzymes of the melanin synthesis pathway, as well as genes involved in cell cycle regulation and cell survival. Somatic amplification of MITF has been identified in melanomas, but MITF activity is mainly altered by its upstream activators and suppressors acting on the transcriptional, post-transcriptional and post-translational levels.

Other genes in melanomagenesis

Other genes causatively implicated in melanomagenesis and progression include KIT, RAC1 and ARID2. KIT encodes for a tyrosine kinase, which is the receptor of the Stem Cell Factor. Upon ligand binding, multiple signalling pathways affecting cell growth, proliferation, survival, and migration are activated. In CM, mutations in KIT occur most commonly in melanomas originating from chronically sun damaged skin and in the acral subtype[118]. The RAC1 gene encodes for a GTPase of the Ras superfamily with important roles in cell motility. A hot spot mutation at P29S, is the result of a C>T transition, consistent with the molecular signature associated with UV damage[18]. The ARID2 gene encodes for a subunit of the switch/sucrose non-fermentable (SWI/SNF) chromatin remodelling complex, a multiprotein complex that alters chromatin structure to regulate gene expression[134]. Recent evidence suggests that components of the SWI/SNF complex, function as tumour suppressors in several types of cancer. In the case of CM, loss-of-function mutations in the ARID2 gene are the most frequent among SWI/SNF enzymes.

CONCLUSION

In this review, we present the main genetic features contributing to the development of CM. Marked advances in dealing with this complex disease have been achieved over the last years, due to the diligent efforts of researchers to shed light on the biological mechanisms involved in melanoma manifestation, assisted by the advent of NGS technologies. Elucidating the mechanisms underlying melanoma biology and progression can enable the development of targeted and immune-related therapeutic approaches. Still, melanoma remains one of the most lethal types of cancer. Additional understanding of the resistance to targeted therapies is crucial, and ought to remain a central aspect of cancer research. The intervention schemes based on combination approaches are the most promising therapeutic ways, in the context of personalised treatment.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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