Enhancing the utility of chromosome 6 and 8 testing in uveal melanoma biopsies

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Background. The aim of this study was to evaluate the significance of testing the gain of chromosome 8 and the gain of chromosome 6 as prognostic markers in histopathological samples of enucleated eyes in with uveal melanoma.
Methods. This is a retrospective study of 54 enucleated eyes. The status of chromosomes 3, 8 and 6 was tested by CISH, and FISH was used in a few samples. A follow-up for the detection of metastases was conducted in all patients. The statistical significance of chromosomal abnormalities as a prognostic factor for the development of metastases was determined.
Results. The study group consists of 54 patients (average age 63 years), 28 men (51.9%) Monosomy 3 together with gain of chromosome 8 was found in 10 samples (18.5%). Both chromosomal abnormalities were detected in 6 (11%) patients. No chromosomal abnormality in 3 or 8 was detected in 21 (38.9%) patients. Abnormalities of chromosome 6 were present in 6 (11%) patients. Progression free survival after 5 years was 33.3% (95% CI 0.0; 83.3) in these patients.
Conclusions. Our findings indicate a correlation between progression-free survival and the presence of changes in chromosome 3 and e 8 in uveal melanomas. The results underline the necessity of testing for both chromosomal aberrations.

Key words: uveal melanoma, chromosome 6, chromosome 8, CISH, FISH, progression free survival

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INTRODUCTION

In adults, uveal melanoma (UM) is the most common malignant metastatic disease. Metastases typically involve the liver and are usually fatal within a year of symptom onset. This is likely due to micrometastases, which probably existed long before metastasis detection by conventional imaging methods[1-4]. There were no effective therapies targeting metastases until several years ago and the mortality rate has remained stable over the last decades for example, there was no change in the 5-year relative survival rate (81.6%) from 1973 to 2008 (ref.[5]). Tebentafusp is a new form of immunotherapy. It belongs to the immTAC group (immune mobilizing monoclonal TCR against cancer). The high-affinity TCR (T cell receptor) binds to an antigen that is presented in a complex with HLA (human leucocyte antigen) on the cell surface. This TCR is fused to a single-chain anti-CD3 antibody fragment[6]. Tebentafusp is only effective for a certain type of HLA, it must be linked to HLA-A*02:01, which is present in about 50% of patients[7]. Tebentafusp is the first drug of this group to enter into clinical practice[8].

Prognostication is an important tool in consideration of these new therapeutic approaches[9]. Chromosomal abnormalities play an important role in this field. Previously, risk factors included tumour location, tumour size, histopathologic cell type or infiltrating lymphocytes. However, these factors alone were insufficient, and it was necessary to assess several factors together[10]. The American Joint Committee on Cancer (AJCC) published the AJCC staging manual which is available for numerous solid cancers, including uveal melanoma. The classification is based on the schema: tumour (T), node (N) and metastases (M). The histopathologic type – spindle melanoma, mixed melanoma and epithelioid melanoma is included as well. According to tumour size, ciliary body involvement, and extraocular extension, uveal melanomas are divided into 4 categories, 17 subcategories and 4 stages. The seventh edition of the manual was published in 2015 (ref.[11]), the eighth edition in 2018 (ref.[12]). The prognostic value of the classification in real clinical practice has been verified by several authors. In a detailed analysis of more than 7000 patients Shields et al showed the prognostic value of this classification (7th edition). The authors showed a strong association between T category and development of metastases, and they found an increasing risk of metastases in all categories (T1–T4) (ref.[13]). Another analysis focused on the anatomic stage. The rate of metastasis was three times greater for stage II, and 9 times greater for stage III (ref.[14]). Similar results were found by AJCC

Ophthalmic Oncology Task Force[11]. However, in a certain number of patients with stage I (5%), metastases developed after 5 years and conversely, 44% of patients with stage III after 5 years had no metastases. The situation was the same with tumour size. In category T1, metastases after 5 years were present in 8% vs. T4 in 51% (ref.[13]).

A turning point in predicting the risk of metastasis development was the use of genetic analysis of tumour samples[15]. An important sign of cytogenetic changes is that they are non-random[16]. The most common abnormalities are monosomy 3, additional copy of chromosome 8 and structural or numerical changes in chromosome 6. Changes in chromosomes 1 or 9 are observed less frequently[17]. Knowing that 50% of patients may develop metastases, it is essential to have a reliable tool to predict this meta process even in the early stages of the disease[18]. These patients at risk could then benefit from early immunotherapy intervention. Chromosomal aberrations could be such a tool.

The objective of this cytogenetic investigation is to validate the importance of the testing of chromosome 8 and 6 as prognostic indicators in histopathological specimens from enucleated eyes afflicted with uveal melanoma. We discuss the validity of these chromosomal abnormalities and their contribution to chromosome 3 testing. We wanted to determine the most appropriate combination of examined chromosomal aberrations.

MATERIAL AND METHODS

This work is a retrospective study of 54 patients after enucleation because of the presence of posterior melanoma or ciliary body melanoma unsuitable for brachytherapy or cyber knife therapy. The study comprised patients who underwent enucleation in the Department of Ophthalmology between 2012 and 2022. Throughout the duration, the patients were monitored at the same institution.

Study group

The samples of enucleated eyes were examined by cytogenetic methods in the Department of Pathology. The monitored clinical-pathological characteristics are listed in Table 1.

Ultrasound (Compact touch, Quantel Medical, France) was used for the setting of the diagnosis and for the measurement of the thickness and diameter (mm) of the tumour. The total size of the tumour for statistical evaluation was calculated by mathematical approximation. We did the oncologic staging every 3 months during the first 2 years and twice a year in the following years. We received information about the death of the patient and about cause of death from the electronic system of the Institute of Health Information and Statistics of the Czech Republic. Based on this data, the occurrence of metastatic-related mortality (disease-specific survival rate) and mortality from other causes was documented.

We determined progression free survival (PFS) based on collected data. Informed consent was obtained from all the participants. Written informed consent has been obtained from the patients to publish this paper. We adhered to the principles of Good Clinical Practice (GCP) in accordance with the Declaration of Helsinki.

Table 1. Characteristics of study group.

		Total (n=54)	Monosomy 3 with gain chr. 8 (n=10)	Disomy 3 without gain chr. 8 (n=44)	*P*
Sex – female		26 (48.1%)	4 (40.0%)	22 (50.0%)	0.730
Age at the time of diagnosis (years)		63 ± 14 67 (56; 71)	62 ± 6 62 (57; 67)	63 ± 15 68 (56; 71)	0.300
Location	Posterior pole	8 (14.8%)	4 (40.0%)	4 (9.1%)	0.099
	Nasal part	15 (27.8%)	3 (30.0%)	12 (27.3%)	
	Temporal part	19 (35.2%)	2 (20.0%)	17 (38.6%)	
	Ciliary body	12 (22.2%)	1 (10.0%)	11 (25.0%)	
Histological cell type	Epithelioid	8 (14.8%)	3 (30.0%)	5 (11.4%)	**0.040***
	Spindle. type A	15 (27.8%)	1 (10.0%)	14 (31.8%)	
	Spindle. type B	10 (18.5%)	0 (0.0%)	10 (22.7%)	
	Mixed	21 (38.9%)	6 (60.0%)	15 (34.1%)	
Thickness of melanoma (mm)		8.3 ± 3.7 8.3 (5.5; 12.0)	9.2 ± 3.9 9.5 (6.0; 12.0)	8.1 ± 3.7 7.9 (5.3; 11.5)	0.448
Size in diameter (mm)		14.6 ± 4.0 14.0 (12.0; 17.0)	15.7 ± 3.6 15.8 (12.0; 19.0)	14.4 ± 4.0 14.0 (12.0; 17.0)	0.377
Total size of melanoma (cm^3)		1.27 ± 1.07 0.96 (0.45; 1.80)	1.53 ± 1.28 1.22 (0.88; 1.38)	1.21 ± 1.02 0.81 (0.43; 1.88)	0.261
Rupture of Bruch's membrane		25 (46.3%)	6 (60.0%)	19 (43.2%)	0.485
Secondary retinal detachment		41 (75.9%)	7 (70.0%)	34 (77.3%)	0.689
Extrabulbar spread		2 (3.7%)	0 (0.0%)	2 (4.5%)	1.000

* Significant ($P<0.05$) difference between categories.

Detection of chromosomal abnormalities

At the Department of Pathology, the collected eye samples were subjected to cytogenetic analysis to detect chromosomal abnormalities. For cytogenetic analysis, archival tissues fixed with formalin and embedded in paraffin were used. Histopathological classification was done on hematoxylin and eosin stained slides according to standard histopathological practice. Fluorescence in situ hybridization (FISH) and chromogenic in situ hybridization (CISH) were used to detect chromosomal abnormalities, namely chromosome 8 gain, chromosome 6 gain and chromosome 3 monosomy.

Fluorescence in situ hybridization analysis was performed on 3-µm sections. Briefly, deparaffinization, protease treatment, and washes were carried out. After pretreatment, the slides were denatured in the presence of 10 µL probe for 10 min at 75 °C and hybridized at 37 °C overnight. Post-hybridization SSC washes were done at 38 °C and the slides stained with DAPI before analysis. The FISH slides were evaluated using a Zeiss Imager Z2 fluorescence microscope with an Axiocam 512 mono camera. ZEN 2.6 software (blue edition) was used. For counting the signals a ×63 objective was used. Hybridization signals were counted in 100 nuclei per sample.

ZytoLight ® SPEC FGFR1/CEN 8 Dual Color Probe and ZytoLight ® SPEC MYC/CEN 8 Dual Color Probe were used to analyze chromosome 8q status. The gain of the long arm of chromosome 8 was defined by amplification of the MYC gene (located in chromosomal region 8q24.21) and copy loss of the FGFR1 gene (located in chromosomal region 8p11.23-p11.22). To mitigate uncertainty, an additional probe, RUNX1T1, located on the long arm of chromosome 8 in the 8q21.3-q22.1 region, was used.

Chromosome 6 was probed with the ZytoLight ® SPEC RREB1/MYB/CEN 6 Triple Color Probe. Gain on the short arm was defined by amplification in regions that map to target sequences in 6p24.3-p25.1.

Identification of monosomy 3 was performed by FISH or CISH. Fluorescence in situ hybridization was used to detect chromosome 3 abnormality using the ZytoLight ® PEC PIK3CA/CEN 3 Dual Color Probe (ZytoVision GmbH, Bremerhaven, Germany). One hundred cells were examined in each case, with a 20% threshold defining chromosome 3 monosomy.

Chromogenic in situ hybridization analysis was done on 3-µm sections. Tissue sections were deparaffinized followed by heat pretreatment and enzyme digestion. Chromosome 3 DIG Probe (Ventana Medical Systems, USA) was applied to each section. The UltraView Red ISH DIG kit detected the CEN3 signal. Counterstaining with hematoxylin II and a bluing agent was performed to enhance contrast. Imaging was performed using a ZEISS microscope, Imager. Z2 equipped with an Axiocam 305 color camera. ZEN 2.6 software (blue edition) was used for red signal analysis, with a cut-off value of 20% defining a monosomy of chromosome 3.

Statistical analysis

Continuous variables were summarized as means and standard deviation and median (25th and 75th percentile), with differences using the Mann-Whitney U test for statistical significance. Categorical variables are presented as absolute and relative frequencies, and evaluated using

Table 2. Characteristics of study subgroup with monosomy 3.

		Total (n=27)	**Chr. 8 abnormalities** (n=6)	**Without chr. 8 abnormalities** (n=21)	***P***
Sex – female		13 (48.1%)	5 (83.3%)	8 (38.1%)	0.077
Age at the time of diagnosis (years)		62 ± 16 66 (51; 71)	62 ± 22 70 (39; 75)	61 ± 14 63 (56; 71)	0.620
Location	Posterior pole	2 (7.4 %)	0 (0.0%)	2 (9.5%)	
	Nasal part	6 (22.2%)	3 (50.0%)	3 (14.3%)	0.130
	Temporal part	12 (44.4%)	3 (50.0%)	9 (42.9%)	
	Ciliary body	7 (25.9%)	0 (0.0%)	7 (33.3%)	
Histological cell type	Epithelioid	4 (14.8%)	1 (16.7%)	3 (14.3%)	
	Spindle type A	8 (29.6%)	3 (50.0%)	5 (23.8%)	0.442
	Spindle type B	6 (22.2%)	0 (0.0%)	6 (28.6%)	
	Mixed	9 (33.3%)	2 (33.3%)	7 (33.3%)	
Thickness of melanoma (mm)		7.6 ± 3.8 7.0 (4.0; 12.0)	9.2 ± 2.7 9.0 (7.2; 12.0)	7.2 ± 4.0 7.0 (4.0; 11.0)	0.160
Size in diameter (mm)		14.1 ± 4.1 14.0 (11.0; 17.0)	17.2 ± 4.5 16.5 (14.0; 20.0)	13.2 ± 3.6 13.0 (11.0; 15.0)	0.057
Total size of melanoma (cm^3)		1.10 ± 0.99 0.72 (0.35; 1.98)	1.71 ± 1.17 1.40 (0.74; 2.79)	0.93 ± 0.89 0.66 (0.30; 1.22)	0.058
Rupture of Bruch's membrane		10 (37.0%)	2 (33.3%)	8 (38.1%)	1.000
Secondary retinal detachment		21 (77.8%)	6 (100.0%)	15 (71.4%)	0.284
Extraocular extension		1 (3.7%)	0 (0.0%)	1 (4.8%)	1.000

Table 3. Survival by gain of chromosome 8 within patients without monosomy 3.

	Disease specific survival		Progression free survival	
	5-year survival (95% CI)	HR (95% CI)	5-year survival (95% CI)	HR (95% CI)
Total (n=27)	93.8 (81.8; 100.0)	-	83.0 (67.5; 98.5)	-
Without abnormality 8 (n=21)	100.0 (100.0; 100.0)	Reference category	95.2 (86.2; 100.0)	Reference category
Abnormality 8 (n=6)	50.0 (0.0; 100.0)	-	33.3 (0.0; 83.3)	-

Table 4. Progression free survival by typology of chromosome 3 and 8 abnormalities and melanoma size in diameter.

	Progression free survival	
	5-year survival (95% CI)	HR (95% CI)
Total (n=54)	64.5 (50.0; 79.0)	-
Size in diameter ≤ 18 mm without monosomy of chr. 3 and without abnormalities of chr. 8 (n=19)	100.0 (100.0; 100.0)	Reference category
Size in diameter ≤ 18 mm with monosomy of chr. 3 without abnormalities of chr. 8 (n=14)	59.7 (28.5; 90.9)	7.66 (0.88; 66.50)
Size in diameter > 18 mm or abnormalities of chr. 8 regardless of monosomy of chr. 3 (n=21)	34.5 (11.0; 58.0)	18.76 (2.40; 146.59)*

*Significant (P<0.05) change in HR (hazard ratio) between categories.

Fisher's exact test. Disease-specific survival and progression-free survival were estimated with the Kaplan-Meier method, and the log-rank test was utilized to determine significant differences in survival rates among groups. Hazard ratios (HR) between groups were estimated using the Cox proportional hazards regression model. A significance level of 0.05 was used for all hypothesis testing.

RESULTS

In this study group 54 patients were included, 28 men (51.9%) and 26 women (48.1%). The average age of the patients was 63 ± 14years. Table 1 provides a detailed description of the study group. The median of the follow-up period was 5 years. Metastases after 3 years were recorded in 25.5% patients, after 5 years in 25.5% (95% CI 13.0; 38.0) as well. After 5 years 37.6% of patients had died (95% CI 22.3; 52.9).

Monosomy 3 together with gain of chromosome 8 we found in 10 (18.5%) patients (Table 1). A subgroup without monosomy 3 and with chromosome 8 abnormality consisted of 6 (11.1%) patients. No abnormality of chromosome 3 nor chromosome 8 was presented in 21 (38.9%) patients (Table 2). We found no statistical significance between basic clinical characteristics and any of the chromosomal abnormalities. The only exception was the association between the spindle cells histological type and the absence of chromosome abnormalities 3 and 8 (P=0.040), Table 2.

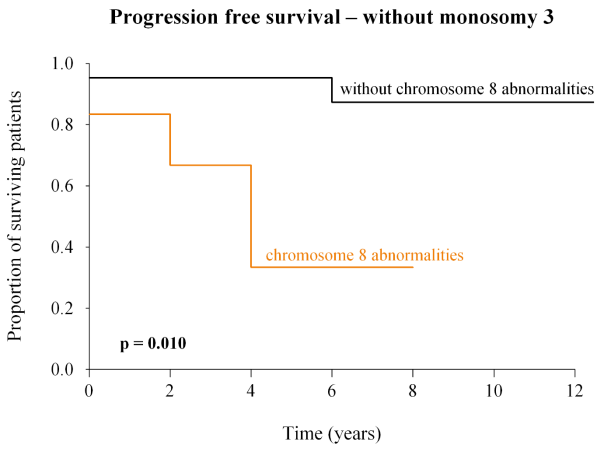

Fig. 1. PFS by abnormalities of chromosome 8 within patients without monosomy 3.

We found a statistically significant connection between progression free survival and the presence of chromosome 8 abnormality in patients without monosomy 3 (33.3; 95% CI 0.00; 83.3), Table 3, Fig. 1.

Patients with a large tumour above 18 mm or with abnormal chromosome 8 (irrespective of monosomy 3) had the worst prognosis in our group, two-thirds of patients had metastases or deaths due to melanoma within five years (Table 4).

DISCUSSION

Metastatic spread is an important issue in patients with uveal melanoma. With the development of new therapies, it is increasingly important to anticipate patients with a high risk of developing metastases.

Various authors have compared the statistical significance of known clinical factors and chromosomal aberrations. An extensive study of aberrations of chromosome 3, 6, and 8 in 1,059 patients was published by Shields et al.[19]. A detailed analysis of chromosomal aberrations (partial or complete monosomy 3, 6p gain / loss, 6q gain / loss, 8p gain / loss and 8q gain / loss) produced 52 individual combinations of chromosomal defects. Kaplan-Meier estimate for melanoma-related metastasis in 5 years for monosomy 3 was 28%, for 6q loss 49%, for 8q gain 35%, respectively. The individual combination of chromosomal aberrations (personalized cytogenetic profile) and the incidence of metastases was evaluated. The Kaplan-Meier estimation was low (5 years) for melanoma-related metastasis for no 3, 6, and 8 chromosomal abnormalities (1%, 1%, 4%) compared with the higher-risk combinations of 3 chromosomal abnormalities. The riskiest combination was 3 complete monosomy, 6 disomy, and 8q gain (39%).

In another work, Shields et al. compared melanoma cytogenetics with some clinical signs of malignant melanoma. In the group with the genetic mutations of all the chromosomes 3,6 and 8 compared to the group without genetic mutation, ocular melanocytosis, ciliary body location and increased basal diameter and thickness of tumour were significantly more frequent[20].

A comparison of individual clinical factors and monosomy 3 and chromosome 8q gain in patients with metastatic process was made by Eleuteri et al. More than 4,000 patients were included and chromosomal aberrations were examined in about 600 patients. The highest hazard ratio of metastasis development was demonstrated in monosomy 3 (HR 4.202, P=0.0000133). Of all clinical factors epithelioid melanoma cells (HR 1.6) had the highest HR, but P was 0.00231, and extraocular extension (HR 1.521, P=0.000255). Thus, this work also confirmed the high predilection value of chromosome 3 aberrations[21]. Damato et al. evaluated chromosomal aberrations 3 and 8 simultaneously with basal tumour diameter and cytologic subtypes and determined in each patient a predictive index for disease-specific mortality by Cox Multivariate Analysis. They count B coefficient basal tumour diameter (0.25), for chromosome 3 loss (1.3) and for epithelioid cells (1.49) (ref.[22]). Vaquero-Garcia et al. created a model for prediction based on clinical and chromosomal information as a Prediction of Risk of Metastasis in UM (PriMeUM). Using clinical and chromosomal information, the accuracy was 85% vs 80% (using chromosomal information only) (ref.[23]).

In our work, we found a statistically significant association between the occurrence of spindle type B melanoma and the absence of chromosomal abnormalities only. When evaluating the statistical dependence of chromosomal abnormalities and some clinical factors such as Bruch's membrane rupture or extraocular extension, our calculations are limited by the size of our study group. Based on the work of Damato[22]. We have looked more closely at the association between the cytogenic profile and tumour size. In our work, we also confirmed that PFS is significantly shortened in patients with tumours larger than 18 mm, as well as in patients with chromosome 8 abnormalities.

The Cancer Genome Atlas (TCGA) is a great project dealing with genetic testing in malignant melanoma of the choroid. The goal of this project is to describe genetic mutations found in human cancers and it is supervised by the National Cancer Institute´s Center for Cancer Genomics and the National Human Genome Research Institute[24-26]. The researchers studied 80 cases of uveal melanoma. They showed the importance of chromosome 3 monosomy and chromosome 8 disomy. By simplifying classification, they divided UM to four molecularly distinct and clinically relevant subgroups according to abnormalities of chromosome 3 and 8. The best prognosis was in patients without any mutation of chromosome 3 or 8 (class A) and the worst prognosis was in patients with a mutation of both chromosomes – class D (ref.[27]). Vichitvejpaisal has validated The Cancer Genome Atlas classification for uveal melanoma in a large study group of six hundred fifty *eight UM patients. They categorized all patients into 4 groups according TCGA (A, B, C, D). The main criterion was chromosome 3 abnormality, disomy 3 – group A, B; monosomy 3 – group C, D. More advanced group revealed increasing risk of metastasis (A – 3% vs. B – 10% vs. C – 25% vs. D – 41%). Comparing group A and D the 5-year hazard ratio for metastasis was 30 (P<0.001) (ref.[28]).

In 2020 Mazloumi published a paper comparing the predictive value of The Cancer Genome Atlas (TCGA) and The American Joint Committee on Cancer (AJCC) (ref.[29]). This work was a retrospective study of 642 UM patients treated with brachytherapy, excluding iris melanoma. Patients were classified according to AJCC 8th edition (4 categories, 17 subcategories and 4 stages). Patients who underwent pre-treatment fine-needle aspiration biopsy for genetic analysis of chromosomes 3 and 8 were classified according TCGA (4 categories). After 5 years TCGA classification showed a higher value for the prediction of metastases. Individual clinical factors were also evaluated as possible predictors of metastatic spread. As predictors, these were shown – tumour thickness, tumour basal diameter, and ciliary body involvement, but their value was less powerful than the TCGA classification as well.

Based on our work, we confirm the importance of examining chromosome 8 aberrations in addition to chromosome 3 abnormalities. The greatest significance of chromosome 8 abnormality is in patients with disomy 3. In these patients, the presence of abnormality on chromosome 8 reverses the good prognosis of the disomy 3. PFS (5 years) according to our observations, is comparable in patients with monosomy 3 and with disomy 3 and abnormalities of chromosome 8.

Abnormalities of chromosome 6 are rare and in our work, we find it is not a predictor of metastatic disease.

We also confirmed the importance of assessing chromosomal abnormalities together with clinical factors, especially tumour size. Monosomy 3 is the most significant but not always the decisive factor.

The next step in understanding the pathogenesis of uveal melanoma is maybe identifying specific genes as predictive factors. A strong correlation with metastatic death in patients with uveal melanoma was observed in BAP1 mutation and class 2 gene expression profile (GEP) (ref.[30]). In a more detailed genetic analysis, these authors found that most tumours with monosomy 3 demonstrated BAP1 alteration. Robertson et al compared uveal and cutaneous melanoma. Uveal melanoma has lower somatic mutational density and a unique set of mutated genes compared to cutaneous melanoma[31]. However, methods for the identification of genes require more expensive and demanding laboratory examinations than chromosomal aberrations, which limit their use in routine clinical practice. A limiting factor of our study group is the small number of patients, although data were collected for ten years, and we are the ocular oncology centre for the whole of Southern Moravia (1 200, 000 citizens).

CONCLUSION

The important process of the future care of the patients with uveal melanoma is the testing of chromosomal abnormalities to predict metastatic spread. Most important is the examination of chromosome 3. Monosomy 3 relates to a higher risk of developing metastases. There is a need to test chromosome 8, mainly in patients without monosomy 3. The presence of aberrations of chromosome 8 overturns the good prognosis of disomy 3. Based on our experience it is not necessary to standardly test chromosome 6 aberrations due to their low incidence and minimal predictable value.

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Ethics approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments. Informed consent was obtained from all individual participants included in the study.

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