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FREQUENCY OF *CLAR* AND *JAK2* MUTATIONS IN SUDANESE CHRONIC MYELOID LEUKEMIA PATIENTS WITH PHILADELPHIA-POSITIVE DISEASE

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ABSTRACT

Objective: It is well-established that myeloproliferative diseases coexist with *CLAR* and *JAK2*. In Ph+ chronic myeloid leukemia (CML), only a few case reports indicate the existence of CLAR, *JAK2V617F*, and *JAK2* exon 12 mutations.

Methods: This study examined *CALR* and *JAK2* mutation profiles in Sudanese Chronic Myeloid Leukemia patients with Philadelphia-positive patients. Blood samples were collected from 100 patients with Ph+ CML chromosomes. Results for the JAK2V617F mutation were confirmed using the TaqMan® Mutation Detection Assay, and the four common mutations on exon 12 and *CLAR* mutations were confirmed using allele-specific PCR (AS-PCR) and Sanger sequencing.

Results: CML patients with *CALR* frameshift mutations were detected in two patients (2%), patients with *JAK2* exon 12 mutations were found in two patients (2%), and patients with *JAK2V617F* mutations made up 4 (4%) of the total CML patients. No significant relationships existed between mutations and age, WBC, RBC, Hb, HCT, or platelet parameters. Patients with *CLAR*, *JAK2* exon 12, and *JAK2V617F* mutations have normal leukocyte counts and lower values compared to triple-negative Ph+ CML, but these differences are not statistically significant (p values for each 0.084, 0.173, and 0.072).

Conclusion: It is conceivable for Ph+ CML and all mutations to coexist.

Keywords: Chronic myeloid leukemia, Philadelphia chromosome, BCR-ABL, CLAR mutation, JAK2V617F mutation.

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INTRODUCTION

Chronic myeloid leukemia (CML) can be distinguished from other forms of classic myeloproliferative neoplasia by the pathognomonic Philadelphia (Ph)-chromosome, which corresponds to translocation t (9;22) and produces the BCR-ABL1 oncogene (MPN). The X-linked glucose 6-phosphate dehydrogenase (G6PDH) polymorphisms in hematopoietic cells of CML female patients carrying GDPDH heterozygosity was investigated in the classic work of Fialkow *et al*. [1]. They showed that the Ph-chromosome develops in a multipotent stem cell and is present in all hematopoietic lineages, including B lymphocytes. Using delicate methods like fluorescent *in situ* hybridization, more study on sorted cells has verified this finding [2,3].

Numerous chromosomal aberrations and a similar G6PDH pattern in a Ph-negative B-lymphoid cell line derived from CML patients suggest a multistep etiology for the illness. These data indicate that at least two processes, one of which causes aberrant pluripotent stem cell proliferation and the other of which induces the Ph-chromosome in the offspring of these cells, are necessary to establish the CML phenotype [4]. The "Fialkow theory" was created as a result, and it postulated that the Ph-chromosome acquisition could result in the development of CML in clonal hematopoiesis with genetic instability. Similar to CML, Ph-negative MPN also arises from multipotent hematopoietic stem cells [5].

The 2005 discovery that the Janus kinase 2 (*JAK2*) gene contributes to polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis led to an improvement in the MPN diagnosis criteria (PMF) [6]. *JAK2*-negative myeloproliferative disorders have been linked to mutations in the calreticulin (*CALR*) and thrombopoietin genes, according to research published in 2013 [7,8].

This study examined chronic myelogenous leukemia patients from Sudan to evaluate their *CALR*, JAK2 exon 12, and *JAK2* exon 14 mutational profiles (CML). The relationship between mutation patterns and clinical and hematologic characteristics was examined by correlation analysis.

METHODS

Patients

One hundred patients with Ph+ CML undergoing therapy at the Radioisotopes Center Khartoum (RICK) in Khartoum, Sudan, were enrolled in this study. The research was conducted at the Radioisotopes Centre Khartoum (RICK) in Khartoum State from August 2020 to December 2022. The availability of PB samples obtained at diagnosis or recurrence and the accumulation of symptoms throughout the follow-up period served as the inclusion criteria for this investigation. Strictly by the 2008 WHO categorization criteria, CML was identified [9]. Each patient had laboratory and clinical data collected. Age, gender, ancestry, hemoglobin level, platelet count, and presence or absence of splenomegaly are all factors in the timing of a diagnosis and the start of treatment. The CEGMR Bioethical Committee gave this work its blessing and assigned it the ethical number 01-CEMGR-Bioeth-2020. The implementation of this study adhered to the Helsinki Declaration. All PB samples had informed consent obtained.

PB hematological examination

Using a fully automated blood analyzer (Sysmex KX-21N, Tokyo, Japan; Sysmex), the hematologic parameters of the samples were determined (Hb, Hct, RBCs, WBCs, and Plt).

DNA extraction and detection of mutations

Genomic DNA was examined in the peripheries (PB) of all patients. The manufacturer's instructions for the QIAGEN kit were followed to extract

DNA. The DNA quality was assessed using a 2000c spectrophotometer using quantitative methods (NanoDrop Technologies, Wilmington, DE). The ratio of absorbances at 260 and 280 nm (A260/A280) was used to determine purity. A pure DNA sample's A260/A280 ratio should be between 1.7 and 1.9.

Quantitative real-time PCR (qRT-PCR) technology (QuantStudio 12K Flex) and the TaqMan® Mutation Detection Assay were used to conduct mutation research on JAK2V617F. Sanger sequencing was then used to confirm the TaqMan® results and determine the type allele of the mutations. JAK2 exon 12 mutations were investigated using multiplex PCR, whereas CLAR mutations were examined using the hotspot method. For polymerase chain reaction (PCR) amplification of the CALR, JAK2 exon 14, and JAK2 exon12, the following primers were used: CALR forward, 5'-CAT TCA TCC TCC AGG TCA AG-3'; CALR reverse, 5'-AGG GGA ACA AAA CCA AAA TC-3'; JAK2 exon 14 forward, 5'-CTC CTC TTT GGA GCA ATT CA-3'; JAK2 exon 14 reverse 5'-GAG AAC TTG GGA GTT GCG ATA-3' JAK2 exon 12 forward, 5'-CTC CTC TTT GGA GCA ATT CA-3'; JAK2 exon 12 reverse, 5'-GAG AAC TTG GGA GTT GCG ATA-3'; K539L, 5'-CAT ATG AAC CAA ATG GTG TTT TCA CTT-3'; N542-E543del, 5'-CAA ATG GTG TTT CAC AAA ATC AGA GATT-3', F537-K539delinsL, 5'-CAT ATG AAC CAA ATG GTG TTA ATC-3'; H538QK539L, 5'-CAT ATG AAC CAA ATG GTG TTT TCA ATT-3'. The amplified 537-bp, 453-bp, 280-bp, and 212-bp fragments, respectively, spanned exons 8 and 9 of CALR and exon 12 of JAK2. The PCR reaction was made of the following: Genomic DNA template 1 µL, 2.5 µL (10×) PCR buffer (50 µL PCR buffer, 1.5 µL MgCl2), 10Mm dNTPs 0.5 µL, Forward primer (200 ng/mL) 0.5 µL, Reverse primer (200 ng/mL) 0.5 µL, 0.1 µL of Taq DNA polymerase (plantiumtaq), and 19.9 µL of deionized distilled water were added. The total volume of the amplification reaction was 25 µL. A 5-minute denaturation step at 94°C was followed by 35 cycles of 94°C for 30 s, 58°C to 64°C for 30 s (depending on the primers), and 72°C for 60 s, with a final 7-min extension step at 72°C. PCR products were purified and sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI 3730 XL automated sequencer (Applied Biosystems) with the primers listed.

Statistical analysis

The mean, standard deviation, and percentage are used to report data. Fisher's exact test and the t-test were used to compare categorical variables, and the Mann–Whitney U-test was used to evaluate continuous variables. SPSS 20.0 was used for the statistical analysis (SPSS, Chicago, IL). p-values of 0.05 or less were considered statistically significant.

RESULTS

Enrolled patients' clinical characteristics

All patients were Sudanese, and their average age was 48 years (range 23–63 years). There were 59 (59%) males and 41 (41%) females. The counts of WBCs, RBCs, hemoglobin, hematocrit, and platelets are presented in Table 1.

CALR and additional mutations

Among 100 Ph+ CML patients, 2 (2%) had CLAR frameshift mutations, 4 (4%) had JAK2 exon 14 mutations, 2 (2%) had JAK2 exon 12 mutations, and 92 (92%) had a triple-negative status for all three mutations (Table 2). One patient (1%) had a typical Type 1 mutation (L367fs*46), and 1 patient (1%) had a Type 2 mutation (K385fs*47) among the CALR frameshift mutations Fig. 1.

There were no significant relationships between *CLAR*, *JAK2V617F*, and *JAK2* exon 12 and age, WBCs, RBCs, Hb, HCT, and platelet parameters at the time of diagnosis. Patients with *CLAR*, *JAK2V617F*, and *JAK2* exon 12 CML mutations have normal leukocyte counts and lower levels than those with triple-negative CML, although these differences are not statistically significant (p values for each: 0.084, 0.173, and 0.072) (Table 3).

CALR mutations and clinical characteristic correlation

Compared to patients with *JAK2V617F* mutations, *CLAR* frameshift mutations were related with younger age, higher hemoglobin levels, higher hematocrit levels, and lower platelet levels in Ph+ CML patients. In CML patients with *CLAR* mutations and *JAK2V617F* mutations, no significant correlation was detected between WBC and RBC numbers. There were slightly more male patients (50%) but fewer patients with *CLAR* mutations than *JAK2V617F* mutations (25%). Compared to patients with *JAK2* exon 12 mutations, *CLAR* frameshift mutations were related with younger age, higher RBC levels, higher hemoglobin levels, and higher hematocrit levels. Comparing Ph+ CML with *CLAR*

Table 1: Clinical and laboratory features of 100 CML patients

Variable	Total MPN (n=100)
Male/female, male%	59/41 (59%)
Age, y (mean±SD)	46.54±9.03
Leukocytes, ×109/L (mean±SD)	84.51±103.96
Erythrocyte, ×109/L (mean±SD)	3.78±0.91
Hemoglobin, g/dL (mean±SD)	11.04±2.19
Hematocrit, % (mean±SD)	33.95±7.64
Platelets, ×109/L (mean±SD)	328.79±240.02

Table 2: The number of mutated diseases

Mutation	No. (%) of Cases (n = 100)
JAK2	6 (6.0)
JAK2V617F	4 (4.0)
JAK2 exon 12	2 (2.0)
CALR frameshift	2 (2.0)
Type 1 mutation	1 (1.0)
Type 2 mutation	1 (1.0)
Triple negative	92 (92.0)

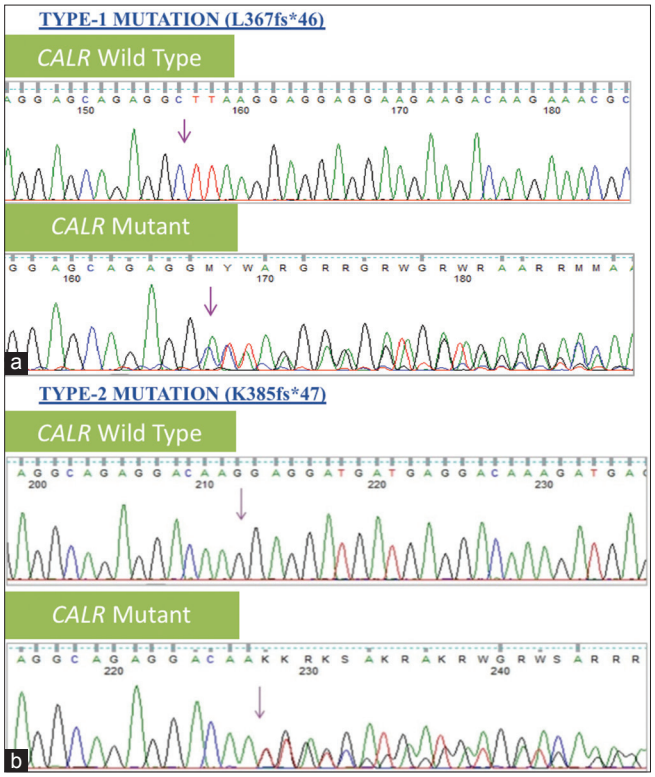

Fig. 1: Sequencing through Sanger for the CALR exon 9 mutation. (a) Normal control samples versus positive samples within frame deletion mutation (L367fs*46), and (b) Normal control samples versus positive samples with in-frame deletion mutation (K385fs*47)

Table 3: Mutations and clinical features correlation

Variable	*CLAR* mutations			*JAK2* exon 14 mutations			*JAK2* exon 12 mutations		
	***CLAR* WT**	***CLAR* mutated**	**p-value**	***JAK2* exon 14 WT**	***JAK2V617F* Mutant**	**p-value**	***JAK2* exon 12 WT**	***JAK2* exon 12 Mutatnt**	**p-value**
Age, y (mean±SD)	46.58±9.08	44.50±7.78	0.390	46.29±9.04	52.50±7.14	0.092	46.48±9.11	49.50±0.71	0.348
Leukocytes, $\times 10^9$/L (mean±SD)	86.12±104.39	5.25±0.49	0.084	86.52±105.23	36.06±52.28	0.173	86.13±104.39	5.00±0.85	0.072
Erythrocyte, $\times 10^9$/L (mean±SD)	3.76±0.91	4.78±0.45	0.069	3.76±0.92	4.19±0.62	0.179	3.78±0.92	3.98±0.67	0.362
Hemoglobin, g/dL (mean±SD)	11.03±2.21	11.45±0.21	0.385	11.07±2.22	10.43±0.94	0.291	11.04±2.21	11.10±0.28	0.470
Hematocrit, % (mean±SD)	33.84±7.76	39.55±0.35	0.144	33.89±7.77	35.53±2.84	0.328	33.90±7.69	36.45±4.03	0.309
Platelets, $\times 10^9$/L (mean±SD)	330.08±242.30	256.50±9.19	0.417	331.31±244.67	268.25±19.14	0.344	331.34±241.67	204.00±77.78	0.229

mutations to patients with *JAK2* exon 12 mutations, no significant correlation between WBC or platelet count was identified. Patients with *CLAR* mutations are slightly more likely to be male (50%) than those with *JAK2* exon 12 mutations (2%). Statistically, however, there was no significant difference between these groups (p=0.156). Unfortunately, the tiny sample size made it challenging to obtain meaningful results. Table 4 presents a summary of the outcomes.

DISCUSSION

The *JAK2V617F* mutation is strongly associated with PV, ET, and PMF, the three main subtypes of BCRABL-negative myeloproliferative disease (MPD). More than 95% of patients with PV and more than 50% of patients with ET and IMF have this mutation [10-13].

Mutation testing, which is important in diagnosing and prognosis of disease, can provide prognostic and other helpful information. For instance, compared to *JAK2* and PMF Type 2 *CALR* mutations, PMF Type 1 *CALR* mutations are associated with a higher rate of survival [14]. *JAK2* mutations are linked to elevated hemoglobin, aging, leukocytosis, thrombotic events, and thrombocytopenia in all MPNs [14,15]. Many molecular techniques assess *JAK2V617F*, *JAK2* exon 12, and *CALR* mutations. Both allele-specific (hotspot mutation evaluation) and sequence-based approaches fall into this category. In this study, Sanger sequencing was utilized to find *JAK2V617F* and *CALR*, and allele-specific PCR was performed to detect known mutations in exon 12. It was previously believed that the *JAK2V617F* mutation and the BCR-ABL translocation were mutually exclusive; however, Kramer *et al*. [16] identified this mutation in a patient with Ph+ CML. This contradicts the findings of Jelinek *et al*. [12] reported the absence of the *JAK2V617F* mutation in patients with a Ph+ CML. Very few such occurrences have been documented since then [17-19]. Patients with Ph+ CML from Boochia *et al*. [17] and Bee *et al*. [19] had a prior history of PV. In contrast, Jalledes *et al*. [19] and Curtin *et al*. [20] reported instances with pre-existing *JAK2V617F*-positive ET that subsequently acquired Ph translocation. Only the patients of Nadali *et al*. [21] and Fava *et al*. exhibited Ph+ CML with a concurrent *JAK2V617F* mutation and no history of MPD [21].

Pahore *et al*. [18] have the first to report the frequency of the *JAK2V617F* mutation in patients with Ph+ CML from Pakistan. About 26.7% of Ph+ CML patients in their research had this mutation. In our study, 4% of patients had this genetic defect. In both studies, no patients had Ph+ CML at baseline. This type of research has not yet been published in the international literature.

The question is how the *JAK2V617F* mutation affects the etiology, illness progression, and prognosis in Ph+ CML. No definitive answer has yet been uncovered. It is considered that the occurrence of this mutation in Ph+ CML may explain the tyrosine kinase inhibitor resistance [21]. As a result, we infer that the presence of the *JAK2V617F* mutation in nearly 4% of Ph+ CML patients and the *JAK2* exon 12 mutations in 2% of Ph+ CML patients in our study suggests that these two disease-specific mutations may coexist.

In 2013, Klampf *et al*. and Nangaria *et al*. described how frequent and exclusive somatic mutations affect exon 9 of the calreticulin (*CALR*) gene [7,8]. *CALR* mutations were mutually exclusive in subsets of *MPL* and *JAK2* individuals without mutations [22]. The influence of *CALR* on prognosis and clinical outcomes needs to be completed. Numerous other studies have described the presence of *CALR* mutations with low frequency in distinct Ph+ CML cases. *CALR* mutations were mutually exclusive in subsets of *MPL* and *JAK2* individuals without mutations. However, this has not been discovered in relation to other hematological illnesses. The emergence of *CALR* gene mutations has altered the Ph+ CML landscape and was originally identified as a somatic mutation in MPN patients in 2013, but lacking alterations in *MPL* or *JAK2* by Klumpf *et al*. [22]. The cytoplasm, endoplasmic reticulum, and cell surface contain CALR proteins. It regulates calcium hemostasis,

Table 4: Laboratory features of 100 Ph+ patients with CML based on their mutation profiles

Variable	Total CML (n=100)	*CALR* Mutated (n=2)	*JAK2* exon14 Mutated (n=4)	*JAK2* exon 12 Mutated (n=2)	Triple Negative (n=92)	p-value (*CLAR* vs *JAK2* exon 14)	p-value (*CLAR* vs *JAK2* exon 12)	p-value (*JAK2* exon 14 vs *JAK2* exon 12)
Male/female, male	59/41 (59%)	1/1 (50%)	1/3 (25%)	0/2 (0%)	56/36(60.6%)	0.007	0.156	0.085
Age, y (mean±SD)	46.54±9.03	44.50±7.78	52.50±7.14	49.50±0.71	46.34±9.10	0.036	0.046	0.002
Leukocytes, $\times 10^9$/L (mean±SD)	84.51±103.96	5.25±0.49	36.06±52.28	5.00±0.85	88.27±105.66	0.450	0.431	0.452
Erythrocyte, $\times 10^9$/L (mean±SD)	3.78±0.91	4.78±0.45	4.19±0.62	3.98±0.67	3.75±0.92	0.062	0.034	0.004
Hemoglobin, g/dL (mean±SD)	11.04±2.19	11.45±0.21	10.43±0.94	11.10±0.28	11.06±7.83	0.025	0.006	0.015
Hematocrit, % (mean±SD)	33.95±7.64	39.55±0.35	33.89±7.77	36.45±4.03	33.83±7.83	0.048	0.003	0.021
Platelets, $\times 10^9$/L (mean±SD)	328.79±240.02	256.50±9.19	268.25±19.14	204.00±77.78	333.88±246.47	0.041	0.122	0.122

apoptosis, and phagocytosis while regulating cell proliferation and promoting the glycoprotein folding [23].

CALR mutations were confirmed in 2 of 100 Ph+ CML patients. Hence, *CALR* mutation Types 1 and 2 was equally detected in Ph+ CML patients. Individuals with heterozygous *CALR* mutations are typically male and younger than those with JAK2 mutations. They typically have low levels of white blood cells and hemoglobin. In such circumstances, myeloid proliferation is more selective to the megakaryocytic lineage, and thrombocytosis is more prominent. In this patient population, longer survival and a decreased incidence of thrombotic complications have been found. The prognostic impact of her *CALR* on Ph+ CML is limited to the Type 1 mutation, but the prognosis of her *JAK2*-mutated PMF is comparable to Type 2 mutations.[24]

While the influence of *CALR* mutations on Ph+ CML is a new scientific finding, the broad impact of *CALR* mutations on Ph+ CML, baseline features, disease progression, patient clinical behavior, and long-term benefits and hazards require additional investigations. Prospective studies should describe the effects of CALR mutations on Ph+ CML, focusing on the homozygous pattern of mutations. Several articles have investigated and detailed *CALR* mutation screening techniques. Some researchers stated that fragment analysis determinations could meet routine diagnostic requirements and contribute to developing real-time PCR detection techniques [25]. These screening methods do not allow for precise classification. Thus, it can be challenging to ascertain the precise size of insertions or deletions using fragment analysis. This is a crucial issue, as our work indicated that in-frame indel polymorphisms could be mistaken as mutations if they are not correctly described. In this study, Sanger sequencing was implemented. This is helpful for identifying mutations by detecting whether an alteration belongs to a clinically relevant kind, that is, Type 1 or 2, or Type 1/2, and distinguishing between polymorphisms and point/nonsense mutations. Nonetheless, it is a significant factor. Mutations may have a crucial role in the diagnostic process.

Rare non-sense mutations have been described, showing the C-terminal deletion of various negatively charged amino acids. Included among these are p.E380X, p.E374X, and p.K391X [26]. Valid testing methods include simultaneous analyses of numerous mutations by next-generation sequencing and sequential testing algorithms. NGS provides an exhaustive investigation of myeloid tumor-associated target genes. Gene inclusion varies by the panel. In addition, the panel's data processing may overlook significant insertions and deletions (particularly *CALR* exon 9 Type 1 alterations) [27]. Due to the comprehensive nature of NGS, it is possible to report harmful variations and poorly described variants of unclear importance at other loci. To ascertain the actual prevalence of the *JAK2V617F* mutation in Ph+ CML, it is recommended to do additional large-scale research. Chronic splenomegaly or an unanticipated hematologic reaction should always raise the potential of an underlying *JAK2*-positive hematopoietic clone after effective treatment for Ph+ CML.

Our knowledge of the molecular structures of Ph+ CML is quickly expanding. Recent research ties other genes and signaling pathways to her Ph+ CML's origin and development [15]. Some of these mutations are not unique to Ph+ CML but represent new biomarkers that can be used to demonstrate clonality or provide additional prognostic information [15]. Several of these mutations are not unique to Ph+ CML but instead represent new biomarkers that can be used to demonstrate clonality or provide additional prognostic data [15]. In terms of processes and accessible knowledge, the complexity of molecular testing is growing. Effective communication between pathologists, physicians, and molecular diagnosticians is crucial for appropriately integrating molecular data with clinical and pathological findings.

CONCLUSION

This is the first study in Sudan to test for *CALR* hotspot and *JAK2* mutations, both of which are present. This fact emphasizes the significance of diagnostic screening for *CALR* and *JAK2* mutations in Ph+

CML patients and those with high platelet counts. Further screening for other predisposing genetic markers may improve the discovery of relevant genetic variants that may aid in understanding disease etiology.

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AUTHOR CONTRIBUTIONS

Conceptualization, E.Y., and A.Y.; methodology, E.Y.; software, E.Y.; validation, E.Y., and A.Y.; formal analysis, E.Y.; investigation, A.Y.; resources, A.Y.; data curation, E.Y.; writing original draft preparation, E.Y., and A.Y; writing—review and editing, E.Y., and A.Y.; visualization, E.Y., and A.Y.; supervision, E.Y.; project administration, E.Y. All authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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This research received no external funding.

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethical approval was obtained from the ethical committee of the Center of Excellence in Genomic Medicine Research (CEGMR) (Bioethical approval code: 01-CEGMR-Bioeth-2020).

INFORMED CONSENT STATEMENT

Informed consent was obtained from subjects involved in the study.

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