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# JAK2 mutation may predict response and guide first line treatment in rheumatoid arthritis

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## Abstract

**Background:** JAK (Janus kinase) inhibitors work by inhibiting the activity of one or more of the enzyme Janus kinase with a therapeutic application for treatment of cancer and inflammatory disorders such as rheumatoid arthritis (RA). We aimed to study impact of JAK2 mutation in serum of rheumatoid arthritis patients on response to first line with conventional synthetic disease-modifying anti-rheumatic drug (csDMARDs) at 3rd month by evaluating DAS28 and ACR response criteria. The study included 85 newly diagnosed rheumatoid arthritis patients and 50 matched controls. Basal JAK2 mutation assessed by PCR in blood samples, TNF- $\alpha$  and IL 6 were measured by ELISA in serum of patient and control groups. All patients started therapy with csDMARDs. Response assessment at 3rd month was evaluated by DAS28 and ACR response criteria. JAK2 mutation was correlated with different clinical and laboratory parameters of patients.

**Results:** Seventeen females (83.5%) and 14 males (16.5%) with age mean  $\pm$  SD (years); (48.7  $\pm$  7.2). Pretreatment JAK2 mutation, TNF- $\alpha$  and IL 6 were significantly high in patients. JAK2 mutation was detected in 45 (52.9%) patients while 40 (47.1%) patients were JAK2 non-mutant. Mutant JAK2 was significantly linked to severity of disease evaluated by DAS28; 14 (70%) of patients with DAS28 ( $\leq$  2.6) were non-mutant JAK2 vs sex (30%) patients mutant JAK2 while 19 (73.1%) of patients with DAS28 ( $>$  5.1) were mutant JAK2 vs 7 (26.9%) patients non-mutant JAK2 ( $P$  0.02). JAK2 mutation found to be significantly correlated with ACR 20, 50, and 70 response criteria; 68.2% of patients with non-mutant JAK2 showed ACR 70 vs 31.8% in mutant group, 52% of patients with non-mutant JAK2 showed ACR 50 vs 48% in mutant group while 31.6% of patients with non-mutant JAK2 showed ACR 20 vs 68.4% in mutant group ( $P$  0.02). JAK2 mutation were more presented in young age patients (mean  $\pm$  SD; 47.1  $\pm$  7.2 vs 50.4  $\pm$  6.9 in mutant vs non-mutant JAK2 patients, respectively with  $P$  0.03). JAK2 mutation was associated with high pretreatment TNF $\alpha$  and IL6 level in serum. Mean  $\pm$  SD of TNF $\alpha$ ; 49.4  $\pm$  41.9 in mutant vs 26  $\pm$  24.4 pg/ml in non-mutant group, with  $P$  (0.003) while mean  $\pm$  SD of IL6; 83.5  $\pm$  56.8 in mutant vs 47  $\pm$  46.9 pg/ml in non-mutant group, with  $P$  (0.002).

**Conclusions:** Adult RA with pretreatment JAK2 mutation significantly showed high disease activity and high pretreatment TNF $\alpha$  and IL6 levels. Patients with JAK2 mutation found to be linked to poor response to 1st line csDMARDs including MTX so they could get more benefit with early introduction of JAK inhibitors as first line monotherapy or when combined with csDMARDs especially those with moderate to severe active RA.

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**Keywords:** Rheumatoid arthritis, JAK2 mutation, First line

## Background

Rheumatoid arthritis is an autoimmune disease with clinical manifestations of chronic synovial inflammation, severe joint damage, and organ damage and is related to significant disabilities and mortalities. Auto-reactive T cells along with cytokines, including tumor necrosis factor alpha (TNF $\alpha$ ), have a significant role in the pathogenic process through accumulation of immune cells and self-perpetuation of inflammatory response. In addition, such cells stimulate matrix metalloproteinase formation and maturation and initiation of osteoclasts resulting in joint and bone destruction [1, 2]. Since joint destruction accompanied by synovial inflammatory reaction appear in early stages of RA, it is essential to manage RA cases at a stage when joint damage is still preventable.

JAK2 mutation has been shown to be linked to myeloproliferative disorders (MPDs) [3] with possibility of measurement in blood sample by PCR [4] and there are several interesting associations between CTDs and MPDs [5]. Interestingly, STAT4 is triggered by Tyk2 and JAK2 in response to interleukin-12 and interleukin-23 resulting in inhibition of signaling and promoting Th1 autoimmune response [6].

The combination of methotrexate (MTX), a synthetic disease-modifying anti-rheumatic drug (sDMARD), and biological disease-modifying anti-rheumatic drug (bDMARDs) which target TNF $\alpha$ , IL-6, and T cells has altered the management of RA. Appropriate clinical remission is now a possible target in several RA cases to stop joint damage. There is evidence that early treatment with sDMARD and bDMARDs is accompanied by improved clinical outcomes and reduced joint destruction and disabilities [7]. Moreover, not only maintaining remission but also inhibition of functional disability and structural alterations are considered long-term target of management by bDMARDs and methotrexate.

However, in daily practice physicians still encounter cases that respond poorly to therapy, cases that no longer respond, and cases that experience adverse events of the drugs.

In spite of advances in bDMARDs, several unmet needs remain. For instance, remission is accomplished and maintained in only low number of bDMARD-managed cases. And even then, upsetting symptoms might persist such as pain, fatigability as well as morning joint stiffness [8]. Moreover, non-responsiveness to bDMARDs along with medication discontinuation because of intolerance

or side effects, highlights the necessity for a novel generation of alternative JAK inhibitors treatments.

Besides, bDMARDs are foreign proteins which have the capacity of induction of immunogenicity and they are administered IV or subcutaneous because of relatively high molecular size.

Research in the previous 20–30 years has revealed that signaling kinases are significant in protein phosphorylation, an essential pathway of intracellular signal transduction [7].

The completion of human genome has permitted recognition of many signaling kinases; currently 518 kinases are identified in human genome and are distributed over 8 families. Among them, JAK belongs to the tyrosine protein kinase family that comprises 90 members. The JAK has significant role in signaling pathways of numerous cytokines, growth factors, and hormones and is incorporated in RA pathogenic processes.

Tofacitinib is selective inhibitor for JAK1 and JAK3; however, it also slightly suppresses JAK2 and Tyk2 [7, 8]. Therefore, it is now used as a pan-JAK inhibitor, being tolerable with great effectiveness for RA.

Such findings augmented the development of other JAK inhibitors including baricitinib, decernotinib, peficitinib, filgotinib, and ABT-494. It is important to note that baricitinib has achieved phase III clinical trials [9].

We hypothesized that RA patients presented with high JAK2 mutation at diagnosis may have poor response to traditional DMARDs

## Aim of the work

Assessment the impact of JAK2 mutation on DAS28, ACR20, ACR50, and ACR70 in RA patients that start treatment with first line csDMARDs. And correlate JAK2 mutation with different laboratory parameters of studied cases.

## Methods

This observational study recruited 85 newly diagnosed RA patients group and 50 control subjects group with matched age and sex. All patients were subjected to complete history taking, complete physical examination (general and local) and laboratory investigations.

Basal JAK2 mutation was measured in blood samples by PCR technique and TNF- $\alpha$  and IL 6 were assessed in serum by ELISA in both patient and control groups. All patients subjected for history taking, physical

examination and laboratory investigation including ESR, CRP, TNF alpha, and IL6 serum levels. Pregnancy and cancer patients were excluded.

#### PCR detection of JAK2 V617F mutation utilizing ASO specific PCR genetic marker

The detection of JAK2 V617F mutation were done utilizing the following primers (JAK2 Reverse: 5'CTGAATAGTCCTACAGTGTTCAGTTTCA 3', JAK2 Forward (specific): 5' AGCATTGTTTAAATTATGAGTATATT 3' and JAK2 Forward (internal control): 5' ATCTATAGTCATGCTGAAAGTAGGAGAAAG 3'). Cycling conditions were 35 cycles with annealing temperature 58.5 °C followed by agarose gel 2% electrophoresis for bands detections [4].

All patients started treatment by conventional synthetic disease-modifying anti-rheumatic drugs (DMARDs) including methotrexate. Response assessment at 3rd month was evaluated by DAS28 and ACR response criteria. JAK2 mutation was linked to various clinical and laboratory parameters of RA cases.

The Disease Activity Score (DAS) and DAS28 were developed for measurement of RA activity in daily clinical practice and in clinical trials. The DAS/DAS28 is a continuous measure of disease activity which combines data from tender swollen joints, acute phase proteins as well as general health. DAS stands for 'disease activity score' and the number "28" indicates the 28 joints which are assessed clinically, HAQ questionnaire assess functional states of joints, X-ray, ultrasonography, and MRI [10].

The ACR20 is a composite measure defined as both improvement of 20% of tender and of swollen joints, and a 20% improvement in three of the next five features: patient's general evaluation, physician assessment, functional ability measure [usually Health Assessment Questionnaire (HAQ)], visual analog pain scale, and erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP). ACR50 and ACR70 are the same instruments with improvement levels defined as 50% and 70% respectively vs. 20% for ACR20 [11].

#### Statistical analysis

The relations between qualitative variables were evaluated by the  $\chi^2$  test or Fisher's exact test, as appropriate. The relations between continuous variables were evaluated by Pearson correlation test. Difference between means of the study parameters were evaluated by the independent sample *T* test. *P* value less than 0.05 was considered significant. Two-sided statistical tests were used in all of the analyses.

#### Results

This study included 71 females (83.5%) and 14 males (16.5%) with age range (36–65 years). There were 50 controls with matches age and gender. Basal ESR and CRP serum values were significantly high among patients (mean  $\pm$  SD; 47.9  $\pm$  17.7 mm/h and 33.2  $\pm$  27.8 mg/L, respectively). Pretreatment TNF-alpha and IL-6 serum measurement were also significantly high in patients than control (mean  $\pm$  SD; 38.4  $\pm$  36.5 pg/ml and 66.3  $\pm$  55.6 pg/ml, respectively). Jak2 mutation was detected in 45 (52.9%) patients by PCR while all controls were non-mutant (Table 1).

Patients with pretreatment mutant JAK2 demonstrated significant high DAS28 (> 5.1) that correlated with severe degree of disease activity. DAS28 > 5.1 found in 19/26 patients (73.1%) vs 7/26 patients (26.9%) in mutant vs non-mutant JAK2, respectively. However, disease remission with DAS28  $\leq$  2.6 was significantly presented in non-mutant JAK2 patients. DAS28  $\leq$  2.6 found in 14/20 patients (70%) vs 6/20 patients (30%) with *P* 0.02 (Table 2).

After 3 months with treatment, composite measure of ACR 70 (which correlate with more improvement in rheumatoid arthritis) was more presented in non-mutant JAK2 patients. ACR 70 was presented in 15/22 (68.2%) non-mutant patients while ACR 20 was presented in 26/38 (68.4%) mutant patients with *P* 0.022 (Table 3).

Patients with mutant JAK2 found to be significantly younger than non-mutant patients (mean  $\pm$  SD; 47.1  $\pm$  7.2 years vs 50.4  $\pm$  6.9 years in mutant vs non-mutant JAK2 patients, respectively, *P* 0.03). However, no difference in basal ESR and CRP level between both JAK2 patients' groups (Table 4).

Pretreatment basal serum TNF.alpha level was significantly high in JAK2 mutant patients (49.4  $\pm$  41.9 pg/ml)

**Table 1** Demographic data, inflammatory markers, mean serum TNF alpha, IL6, and gene mutation of RA patients and control subjects

		Patients	Control
Sex <sup>a</sup>	Male	14 (16.5%)	13 (26%)
	Female	71(83.5%)	37 (74%)
Age years	Range	36–65	33–61
	Mean $\pm$ SD	48.7 $\pm$ 7.2	42 $\pm$ 6.1
ESR mm/h	Mean $\pm$ SD	47.9 $\pm$ 17.7 <sup>a</sup>	11 $\pm$ 4
CRP mg/L	Mean $\pm$ SD	33.2 $\pm$ 27.8 <sup>a</sup>	6 $\pm$ 5.2
TNF-alpha pg/ml	Mean $\pm$ SD	38.4 $\pm$ 36.5 <sup>a</sup>	19.2 $\pm$ 5.4
IL-6 pg/ml	Mean $\pm$ SD	66.3 $\pm$ 55.6 <sup>a</sup>	13.1 $\pm$ 2.7
JAK2	Mutant	45 (52.9%)	0 (0%)
	Non-mutant	40 (47.1%)	50 (100%)

<sup>a</sup> All patients are newly diagnosed at time of study inclusion

**Table 2** DAS28 scores of mutant and non-mutant JAK2 RA patients

DAS28	Non-mutant JAK2	Mutant JAK2	X	P
Remission ( $\leq 2.6$ ) (20 patients)	14 (70%)	6 (30%)	9.24	0.02
Low disease activity ( $\leq 3.2$ ) (24 patients)	13 (54.2%)	11 (45.8%)		
Moderate disease activity (3.2–5.1) (15 patients)	6 (40%)	9 (60%)		
Severe disease activity ( $> 5.1$ ) (26 patients)	7 (26.9%)	19 (73.1%)		

% in rows

**Table 3** ACR composite measure of mutant, non-mutant JAK2 RA patients

ACR	Non-mutant JAK2	Mutant JAK2	X	P
ACR 20 (38 patients)	12 (31.6%)	26 (68.4%)	7.8	0.022
ACR 50 (25 patients)	13 (52%)	12 (48%)		
ACR 70 (22 patients)	15 (68.2%)	7 (31.8%)		

% in rows

**Table 4** Age, ESR, and CRP levels in patients groups

	Patients	Mean $\pm$ SD	T	P
ESR mm/h	Non-mutant JAK2 (40)	46.2 $\pm$ 20.2	0.8	0.4
	Mutant JAK2 (45)	49.4 $\pm$ 15.2		
CRP mg/L	Non-mutant JAK2 (40)	37.7 $\pm$ 32.2	1.4	0.1
	Mutant JAK2 (45)	29.2 $\pm$ 22.7		
Age years	Non-mutant JAK2 (40)	50.4 $\pm$ 6.9	2.1	0.03
	Mutant JAK2 (45)	47.1 $\pm$ 7.2		

vs non-mutant patients ( $26 \pm 24$  pg/ml) with  $P$  0.003. Also, pretreatment basal serum IL6 was significantly high in JAK2 mutant patients ( $83.8 \pm 56.8$  pg/ml) vs non-mutant patients ( $47 \pm 46.9$  pg/ml) with  $P$  0.002, (Table 5).

## Discussion

Rheumatoid arthritis is a chronic inflammatory disorder influencing synovial joints [12]. Furthermore, significant organ damage can occur in RA including heart [13], lungs, skin, eyes, kidneys, and vessels. It is characterized by aberrant innate, cellular, and humoral immune responses [14]. Therefore, abnormal proliferation causes

abnormal survival of activated T cells, B cells, mast cells, neutrophils, macrophages, antigen-presenting cells [15], and synovial tissue fibroblasts [16] that are the principal cellular hallmarks of rheumatoid arthritis.

In synovial joint, the membrane synovium undergoes hyperplasia resulting from activated migration and adhesions of immune cells as well as non-immune cells triggered by different chemokines and adhesion molecules [17]. Besides, the considerably high concentrations of pro-inflammatory cytokines including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-7, IL-8, IL-12/IL-23, IL-15, IL-17, IL-18, IL-32, and IFN- $\gamma$ , along with growth factors, like FGF-2 and vascular endothelial growth factor released predominantly via synovial-like fibroblasts and macrophage, were found to be vital for clinical progression of rheumatoid arthritis with damage of cartilages and erosions of subchondral bones being the main features resulting in joint damage [18]. Therefore, the alterations occurring in joints as a result of factors like inhibition of cartilage-derived ECM formation, enhanced apoptosis of chondrocytes, synovial tissue 'apoptosis resistance' [19], and an enhanced MMP gene expression as well as disintegrin expression are considered the essential components of RA disease [20].

Many signal transduction pathways were reported in rheumatoid arthritis progress. For instance, though IL-1 $\beta$  was found to primarily initiate stress-activated, mitogen-activated protein kinase (SAPK/MAPK) pathway and IL-6 and IFN- $\gamma$  primarily initiate Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, evidence indicates initiation of MAPK signaling via IL-6 [21] and IFN- $\gamma$ . Notably, though tumor necrosis factor alpha was noted to predominantly initiate SAPK/MAPK pathway [22], tumor necrosis factor

**Table 5** Mean serum levels of TNF alpha, IL6 in mutant and non-mutant JAK2 RA patients

	JAK2	N	Mean	Std. deviation	Std error of mean	t	P
TNF.alpha pg/ml	Non-mutant JAK2	40	26	24.4	3.8	3.01	0.003
	Mutant JAK2	45	49.4	41.9	6.2		
IL6 pg/ml	Non-mutant JAK2	40	47	46.9	7.5	3.1	0.002
	Mutant JAK2	45	83.8	56.8	8.4		

alpha could as well initiate JAK/STAT as evident by finding of the study that demonstrated that recombinant human (rh)-TNF- $\alpha$  resulted in STAT3 protein phosphorylation by human chondrocytes in vitro with no alteration of STAT3 content [23]. Importantly, initiation of JAK via IL-6 resulted in triggering SAPK/MAPK pathway and PI3K/Akt/mTOR signaling through 'crosstalk', while PI3K/Akt/mTOR pathway was linked to abnormal survival of non-immune cells as well as immune cells in rheumatoid arthritis [24].

The vital role of JAK/STAT initiation in RA was also proven after FDA approved JAK3-selective small molecule inhibitor (SMI), tofacitinib, as a treatment for rheumatoid arthritis [25]. Definitely, efficacious integration of tofacitinib into the armamentarium of rheumatoid arthritis treatments has facilitated the development of JAK1-selective, JAK2-selective, TYK2-selective, and pan-JAK SMIs for rheumatoid arthritis.

In this study, JAK2 mutation was assessed in serum of RA patients before treatment by PCR technique and we evaluate its impact on response to first line csDMARDs by correlation with DAS28, ACR20, ACR50, and ACR70. Also, JAK2 mutation was correlated with basal TNF alpha, IL6, and clinicopathological features of RA patients.

The MPDs are known precancerous lesions and frequently change to acute myeloid leukemia (AML) that is challenging to manage and commonly results in death and there is a strong evidence of the role of JAK2/STAT pathway in this MPDs progression and the advances in JAK inhibitors as first line treatment in MPD [26].

There is also an identified correlation between STAT polymorphism and susceptibility to rheumatoid arthritis, systemic lupus erythematosus as well as primary Sjogrens syndrome [27]. Interestingly, STAT is initiated by Tyk2 and JAK2 in response to IL-12 and IL-23, with a consequent down-stream signaling and promotion of Th1 triggered autoimmune response [6].

Another interesting link between MPDs and CTDs is that family members have the ability to transform into each other. This seems strange to clinicians from other specialties however for a rheumatologist, transformation of systemic lupus erythematosus to mixed connective tissue disease (MCTD) is well identified as is MCTD into scleroderma. Likewise, SS commonly occurs in association with other CTDs however might precede their onset.

In a study of 617 cases having primary myelofibrosis (MF), the median overall survival (OS) was not better for patients with JAK2 V617F mutation comparing to other mutation in studied patients ( $P < .001$ ). The 10-year cumulative incidence of leukemia transformation was higher for those with JAK2 mutation [28] indicating that JAK2 mutation usually associated with poor

prognosis. Ruxolitinib is a potent and selective JAK2 inhibitor that was approved as a therapy for intermediate-risk or high-risk MF, based on the results of phase III studies (COMFORT-I and COMFORT-II) [29, 30].

The pooled analysis of COMFORT-I and COMFORT-II studies showed that cases managed with ruxolitinib in first line setting had prolonged OS. Long-term use of ruxolitinib was accompanied by decrease in JAK2 allele burden [31, 32]. In COMFORT-I study, more than half of reductions in JAK2 allele burden were seen in 28 cases; 20 of them met the criteria for partial molecular response (PMR) while 6 cases had JAK2 allele burden values below quantifiable limit, meeting the criteria for complete molecular response (CMR).

Our study reported that JAK mutation is correlated with poor response to treatment defined by ACR 20, ACR50, and ACR70 with first line traditional therapies. Looking for the results of a randomized trial involving 958 cases with moderate to severely active RA who had not administered methotrexate or therapeutic doses of methotrexate. The RA cases who administered tofacitinib were considerably more likely to achieve ACR20 and ACR70 after 6 months (71 and 76 vs 51% and 26 and 38 vs 12%, respectively). Clinical benefit was largely sustained after 24 months (ACR20 of 64 and 64 vs 42% and ACR70 of 34 and 38 vs 15%) [33]. So, selecting moderate and severe RA patients with JAK mutation to start with JAK inhibitor may improve the outcome.

Ruxolitinib is a JAK1/JAK2-selective Jakinib [34] that was approved as a treatment of MPDs and psoriasis. As stated by Gadina, developing JAK SMIs which target > 1 JAK does not seem to be 'a problem' [35]. Being generally safe with good tolerability in healthy individuals and RA cases, a study of ruxolitinib demonstrated that p-STAT3 suppression was associated with plasma drug concentrations [36]. In a trial by Williams and his colleagues [37] involving active rheumatoid arthritis cases, an ACR70 response criteria was accomplished in 33% of the cases administering ruxolitinib in comparison to none of the persons receiving placebo.

Ruxolitinib-related suppression of JAK1/JAK2 decreased the blood concentrations of IL-6 and CD-40. Besides, ruxolitinib suppressed p-STAT3 *ex vivo*, i.e., blood cells from rheumatoid arthritis cases. A study by Menet et al. [38] confirmed that JAK1 had an important role in both transduction of common  $\gamma$  chain cytokines and in IL-6 signaling. And these data could emphasize the benefit of JAK inhibitors as first line treatment in RA cases particularly those with moderate or severe activity.

**Baricitinib** is a small-molecule, orally administered, JAK-1 and -2 inhibitor, and in a clinical trial involving

588 cases found that **baricitinib** (4 mg daily taken orally) monotherapy was superior to MTX alone in the proportion of cases achieving an ACR20 response at week 24 (77 vs 62%) [39]. Greater improvement in disease activity and physical function for cases receiving baricitinib (alone or with MTX) was seen compared with MTX alone as early as week 1.

This could explain the poor response of RA patients with mutant JAK2 to traditional lines of therapy and may open new era for tailoring management of RA cases based on RA activity and biological profile.

Decernotinib is a JAK3-selective reversible SMI which is orally given [40]. Decernotinib was found to be effective as a therapy for RA [41]. Its clinical efficiency was shown through improved ACR criteria and DAS28-CRP in comparison to placebo [41]. Also, decernotinib was also assessed for its influence upon JAK/STAT-triggered signaling.

Filgotinib is a selective JAK1 inhibitor [42]. Studies on filgotinib showed selectivity for JAK1 vs JAK2 of nearly 30-folds [43] and the capability of filgotinib to suppress  $T_H1/T_H2$  differentiation and to some extent  $T_H17$  differentiation. Moreover, filgotinib decreased arthritis progression in animal collagen-induced arthritis reflected by decreased paw swelling, decreased cartilages and bones damage, and decreased pro-inflammatory cytokines. Notably, filgotinib effectiveness was comparable in collagen-induced arthritis with etanercept, a TNF biologic.

In a phase IIb trial involving 283 rheumatoid arthritis cases, filgotinib used alone effectively improved RA manifestations [44]. Also, no reported infections or tuberculosis was revealed. In another study [45], authors revealed that filgotinib plus MTX showed a rapid onset of activity, was tolerated and treated RA manifestations.

## Conclusions

Adult RA mutations with JAK2 detected before treatment showed substantial high activity in the disease and high levels of basal TNF $\alpha$  and IL6. In patients with JAK2 mutation, csDMARDs like MTX are found to be linked to poor response so that they can gain greater benefit by early initiation of JAK inhibitors such as first-line monotherapy or in conjunction with csDMARDs, particularly those with moderate to severe active RA.

## Abbreviations

RA: Rheumatoid arthritis; JAK: Janus kinase; MPN: Myeloproliferative disorders; CTDs: Connective tissue diseases; JAK2: Janus kinase 2; MTX: Methotrexate.

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## Authors' contributions

YA: conceptualization, methodology, software. MS: writing—reviewing and editing. AE: supervision, software, validation, interpretation of data. YS: project administration; resources, writing—original draft preparation. All authors have read and approved the final manuscript.

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## Availability of data and materials

All data availability for this work are available upon request to corresponding author.

## Declarations

### Ethics approval and consent to participate

All procedures were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Accepted proposal code by Institutional Research Board (IRB), Faculty of Medicine, Mansoura University: R.20.11.1075 - 2020/11/16. Clinicaltrials.gov registration date: 8/12/2020, code: NCT04667988. Written consent was obtained from entire participants in this study.

### Consent for publication

Consent for publishing patient details obtained.

### Competing interests

All authors declare that they have no competing interests.

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