Determination of the diagnostic/prognostic significance of CaMKK2 in postmenopausal diabetic osteoporosis patients treated with Pioglitazone (thiazolidinedione), Dapagliflozin (SGLT2 inhibitor)

Introduction and Background

Bone health in diabetic women declines with age, and it is reported that the risk of osteoporotic fractures is twice higher in postmenopausal diabetic women than in women without diabetes. Calcium mediated cellular functions are primarily effected through a group of kinases termed as calcium/calmodulin protein kinases (CaMKs) (1). Of the calcium calmodulin kinase kinases (CaMKKs), CaMKK α/I and CaMKKβ/2, CaMKK2 is recognized to play an important role in calcium dependent homeostatic mechanisms (2). Functionally, CaMKK2 coordinates signaling mechanisms related to energy balance, inflammation, and glucose homeostasis (3). Further, as an upstream kinase of adenosine monophosphate kinase (AMPK) CaMKK2 plays a pivotal role in modulating insulin sensitivity, glucose uptake, and thereby diabetic pathogenesis (4),(5). Taken together, in the past decade several studies have documented the role of CAMKK2 in diabetes (6),(7) and recent studies have brought forward the functional association of CaMKK2 in bone health, bone metabolic functions and diabetes. In a parallel track, the Thiazolidinediones (TZDs) and SGLT2 inhibitors class of anti-diabetic drugs are reported to promote bone resorption and resultantly increase the risk of secondary osteoporosis in elderly female patients. Although the peroxisome proliferator-activated receptor gamma **(**PPAR-γ) mediated mechanisms and phosphate resorption, increase in PTH are the primarily identified mechanisms for the osteoporotic effects of TZDS and SGLT2 inhibitors respectively, till date the interconnected/interrelated CaMKK2 mechanisms has not been elucidated. Hence, the present study aims to delineate the modulatory role of CaMKK2-AMPK-PI3K/Akt, CaMKK2-IHH-PPARγ signaling mechanisms in normal, diabetic and diabetic postmenopausal patients treated with the thiazolidinedione, Pioglitazone and the SGLT2 inhibitor Dapagliflozin by means of gene and protein expression studies.

Year I: Aug 2018-Aug 2019
Study Objectives and Methods:

Determination of CaMKK2 and its target gene expression (IHH, SHH, SMO, PTC1, GLI3) and the Pioglitazone, Dapagliflozin (PPAR-γ, PI3K) in the proposed study groups. The study was conducted in AHRC in compliance with the regulatory protocols and the control and experimental groups included 5 participants/patients in the following 8 groups.

(a) Healthy volunteers

(b) Postmenopausal non-diabetic healthy volunteers (PM)

(c) Postmenopausal diabetic patients (PM DM)

(d) Postmenopausal diabetic patients with osteoporosis (PM DM + OP)

(e) Postmenopausal diabetic patients without osteoporosis treated with Pioglitazone (PM DM +Pio)

(f) Postmenopausal diabetic patients with osteoporosis treated with Pioglitazone (PM DM+OP +Pio)

(g) Postmenopausal diabetic patients without osteoporosis, treated with Dapagliflozin (PM DM + Dapa)

(h) Postmenopausal diabetic patients with osteoporosis, treated with Dapagliflozin (PM DM +OP+ Dapa)

Owing to the fact that very limited literature pertains to CaMKK2 expression in blood cells, the first phase of the study was planned to understand the expression of CaMKK2 in the monocytes and PBMC (peripheral blood mononuclear cells). Peripheral blood samples from healthy volunteers was collected and monocytes (in 1ml of blood) and PBMC (6ml of blood) were isolated. Monocyte isolation was carried out utilizing a monocyte isolation demo kit (Stem cell Technologies C.No-15018, Canada) and PBMC was isolated using standard reference protocols.

Monocyte isolation

1ml of blood was mixed with 50 µl cocktail containing magnetic beads coated with tetrametric antibody complexes, mixed well, incubated for 5 minutes, and treated with 3.5ml of PBS (pH 7.8). The mixed tube was placed in the magnetic separator for 5 minutes and the monocytes in suspension was transferred to the another tube. The beads were washed twice and centrifuged at 1500rpm for 5 minutes, and the monocyte containing cell pellet was diluted in PBS-EDTA.

PBMC isolation {Panda, 2012 #19}

- 6 ml of blood samples collected in EDTA vials were mixed well
- 7ml of Ficoll Histopaque (Sigma- Aldrich, C.No-1077,UK_) was taken in a 15ml centrifuge tube
- Using a 1 ml micro pipette the blood samples were slowly, gently layered on the surface of the Ficoll Histopaque.
- The layered blood sample and Ficoll Histopaque was centrifuged at 1800rpm for 30 mins.
- The Whitish buffy coat (about 1 ml) (PBMCs) formed in the interphase between histopaque and medium was gently aspirated without delay.
- The cells were washed (centrifuged at 1800 rpm for 10 min) twice with 10 ml of sterile DPBS (Sigma Aldrich C.No-SH 30028,southloga,Utah)

1. RNA isolation and cDNA synthesis
   The monocytes, PBMC cells were counted and RNA was isolated using RNA isolation kit (Cat No-74104, Qiagen, India) and cDNA was synthesized using a cDNA synthesis kit (Promega 2step q-RTPCR C-No A6010, USA) according the instructions of the manufacturer.

Relative expression of the mRNA transcripts for CaMKK2, IHH, SHH, GLI3, SMO, PTC PPAR-γ and PI3K was studied using q-RTPCR (Agilent Biosystems) using the following list of primers

S. No	Gene/protein Name	Primer information
1	CAMKK2	Fwd: GCAGTGACGCGCTCCTCTCCAA
		Rev: TCCGCTCGTCCATGAATGGGCA
2	PI3K	Fwd: CACCTGAACAGACAAGTAGAGGC
		Rev: GCAAAGCATCCATGAAGTCTGGC
3	IHH	Fwd: CTA CGC CCC GCT CAC AAA G
		Rev: GGC AGA GGA GAT GGC AGG AG
4	SHH	Fwd: ACT GGG TGT ACT ACG AGT CCA AGG
		Rev: AAA GTG AGG AAG TCG CTG TAG AGC
5	SMO	Fwd: CAG GAC ATG CAC AGC TAC ATC G
		Rev: CCA CAA AGA AGC ACG CAT TGA C
6	GLI3	Fwd: CCT CAA AGC GGG CCG CCT GC
		Rev: CAG GTT GTTGTT GGA CTG TGT GC
7	PTC	Fwd: CCA TGT TCC AGT TAA TGA CTC
		Rev: ACA TCA TCC ACA CCA ACA
8	PPAR-γ	Fwd: CCC TGG CAA AGC ATT TGT AT
		Rev: AAT CCT TGG CCC TCT GAG AT

The $2^{-\Delta\Delta Ct}$ values were derived with reference to PM healthy control groups. All experiments were repeated as triplicates.

Results and Discussion

As a pivotal molecule regulating critical physiological functions associated with insulin resistance, glucose intolerance and obesity, CaMKK2 has long been evidenced to participate in the molecular signaling cascades that promote diabetic pathogenesis (4, 5). On a parallel note research evidences in the past five years have demonstrated that CaMKK2 is functionally associated with bone health and bone metabolic functions (8, 9). Based on such evidences and taking into account that CaMKK2 plays a pivotal role in diabetes, osteoporosis, and that inhibition of CaMKK2 utilizing STO-609, promotes fracture healing (10), we hypothesized that CaMKK2 is differentially modulated in postmenopausal, postmenopausal diabetic and postmenopausal diabetic osteoporotic conditions, and may serve to predict osteoporosis, risk of fracture in the these patients. In conjunction we had proposed to examine the (1) the relative gene expression of CaMKK2, its downstream targets from the blood cells of the control and

experimental group patients during the first funded year of the study (2) protein expression and modulation of CaMKK2, its downstream targets from the control and experimental group patients during the second funded year.

Optimization/Determination of the expression of CaMKK2 in monocytes, PBMC.

Based on the availability of literature citing that CaMKK2 expression studies in myeloid cell lineage monocytes/ cultured monocytes (11, 12) and very few reports on CaMKK2 expression in lymphoid cells, we had proposed to examine CaMKK2 in the monocytes isolated from the blood cells of patients. Considering the tediousness associated with the isolation of monocytes, ascertaining the purity of the population and the need for a substantial amount of monocyte cells for the experimental studies, we first optimized/examined the

1. The gene expression of CaMKK2 in the monocytes isolated from healthy volunteers in comparison to the PBMC (monocytes and lymphocytes) from the healthy volunteers/patients.

2. As indicated in Fig.1 we observed that CaMKK2 expression levels from PBMC isolates were equal to that of the expression in monocytes, and we were able to consistently reproduce the data. Hence, based on the results obtained, and the associated cost factors we strategized to utilize PBMCs for the entire experimental studies.

Fig.1: CaMKK2 expression in Monocytes and PBMC of healthy volunteers/patients

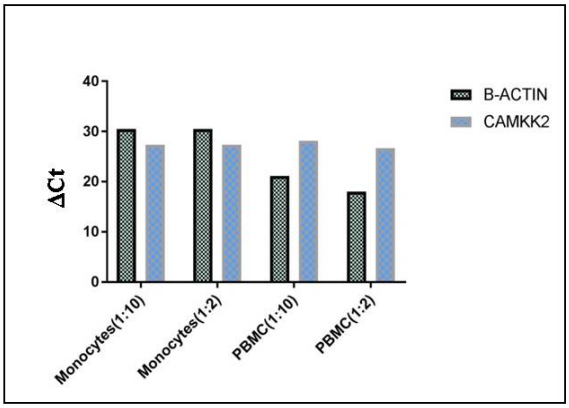


Fig. 1: The ΔCt values for CaMKK2 and β-actin in different dilutions for monocytes and PBMCs.

With the optimization in PBMCs we proceeded to determine the gene expression patterns for CaMKK2 and its associated targets (IHH, SHH, GLI3, SMO, PTC) and the Pioglitazone, Dapagliflozin associated targets PPARG, PI3K, in the control participant/patient groups [healthy, postmenopausal healthy (PM H), postmenopausal diabetic (PM DM), postmenopausal diabetic patients treated with the Pioglitazone (PM DM+Pio), postmenopausal diabetic osteoporosis patients treated with the Dapagliflozin (PM DM+Dapa)] and the study groups [postmenopausal

diabetic osteoporosis (PM DM +OP), postmenopausal diabetic osteoporosis patients treated with Pioglitazone (PM DM +OP+Pio), postmenopausal diabetic osteoporosis patients treated with Dapagliflozin (PM DM+OP+Dapa), with reference to the cell specific reference gene, β2 microglobulin.

Based on the data obtained, the relative gene expression for the target and reference gene β-2 microglobulin was further obtained utilizing quantitative PCR methods as described in the methods section. The ΔCt values obtained for the reference and experimental groups are presented as XY scatter plots in Fig.2, Fig. 3 with the SEM values and it can be observed that variations/errors were minimal and the data obtained was reliable for interpretation.

Fig. 2: CaMKK2 expression in study groups

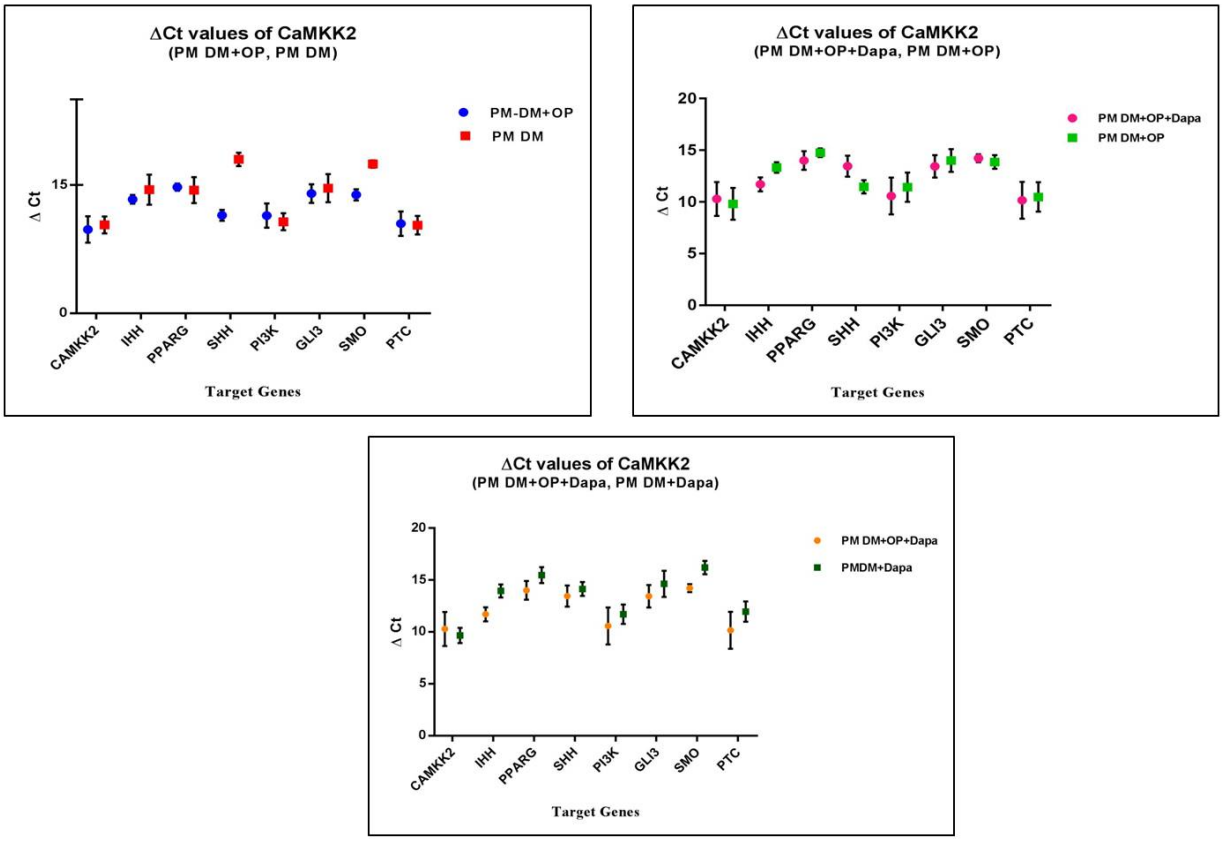


Fig.2: The ΔCt values for CaMKK2 target in the PM DM+OP, PM DM, PM DM+OP+Dapa, PM DM+OP, PM DM+OP+Dapa and PM-DM+Dapa are presented

Fig. 3: CaMKK2 expression in the study group patients

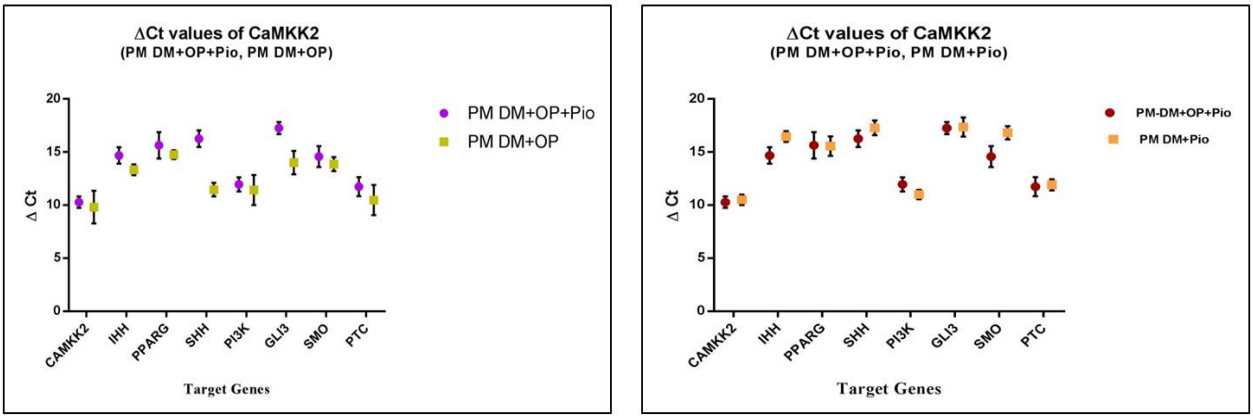


Fig.3: The ΔCt values for CaMKK2 target in the PM DM+OP+Pio, PM DM+OP; PM DM+OP+Pio, PM DM+Pio groups are presented.

We further analyzed the obtained data in detail by means of deriving the ΔΔct, $2^{-\Delta\Delta ct}$ values of the experimental study groups in comparison to the PM healthy group.

<u>CaMKK2, IHH, SMO, PTC and GLI3 in postmenopausal diabetes osteoporosis and the added risk of secondary osteoporosis in postmenopausal diabetic osteoporotic patients treated with Pioglitazone, Dapagliflozin.</u>

Earlier studies assessing the functional role of CaMKK2 reveal that inhibition of CAMKK2 prevents osteoporosis in adult mice by promoting osteoblast formation, and stalling osteoclast differentiation (8). Subsequent studies had also indicated that inhibition of CaMKK2 promotes bone mineral density, and henceforth reverses age related decline of bone mass in adult/senile mice (9). As mentioned earlier, interesting evidences pinpoint that inhibition of CaMKK2 utilizing STO-609 enables fracture healing and endochondrial ossification by modulating the hedgehog signaling component, IHH (Indian hedgehog). As IHH is another reported participant in T2DM (10) like CaMKK2, we hypothesized that these modulations may be reflective in the blood cell population too and proceeded to determine its efficacy in serving as a marker for predicting osteoporotic changes in the postmenopausal diabetic patients treated with TZDs and SGLT2 inhibitors. As presented in Fig.4, Fig.5 the study results indicate that

1. The fold change for CaMKK2 levels are significantly higher in the PM DM+OP, PM DM+Dapa group when compared to the PM healthy groups and the Pioglitazone groups indicating that osteoporotic patients and PM DM patients treated with SGLT2 inhibitors may present higher levels of CaMKK2. Interestingly, the PM DM+OP+Dapa treated group did not present equal or higher fold changes, indicating the possibility that cross talk mechanisms between CaMKK2 and the SGLT2 inhibitor mediated modulations may prevail.

2. On the other hand, CaMKK2 expression was only slightly higher in the PM DM+ OP+ Pio than the PM DM+Pio groups. The reflection of these changes in postmenopausal osteoporotic conditions could be better evaluated utilizing reporter assays and other associated techniques.

To our knowledge this is the first study examining the mRNA expression of CaMKK2 in PBMCs and we report here that although CaMKK2 is of lower abundance in comparison to other signaling moieties, osteoporotic patients may exhibit elevated levels of CaMKK2. Taking into account the complex regulatory mechanisms of CaMKK2 and SGLT2 inhibitors, further detailed analysis may bring to light novel, and essential signaling cascades relevant to the pathophysiology of osteoporosis. We then looked at the signaling targets of CaMKK2 that have been associated with endochondrial ossification of bones, the hedgehog signaling molecules (IHH, SHH, SMO, PTC1 and GLI3) to assess/understand their presence and relevance in the current context. The results indicate that the fold change corresponding to the expression levels of IHH, SHH, SMO, PTC1 and GLI3.

Fig.4: Relative gene expression of CaMKK2, IHH and SHH in the study groups

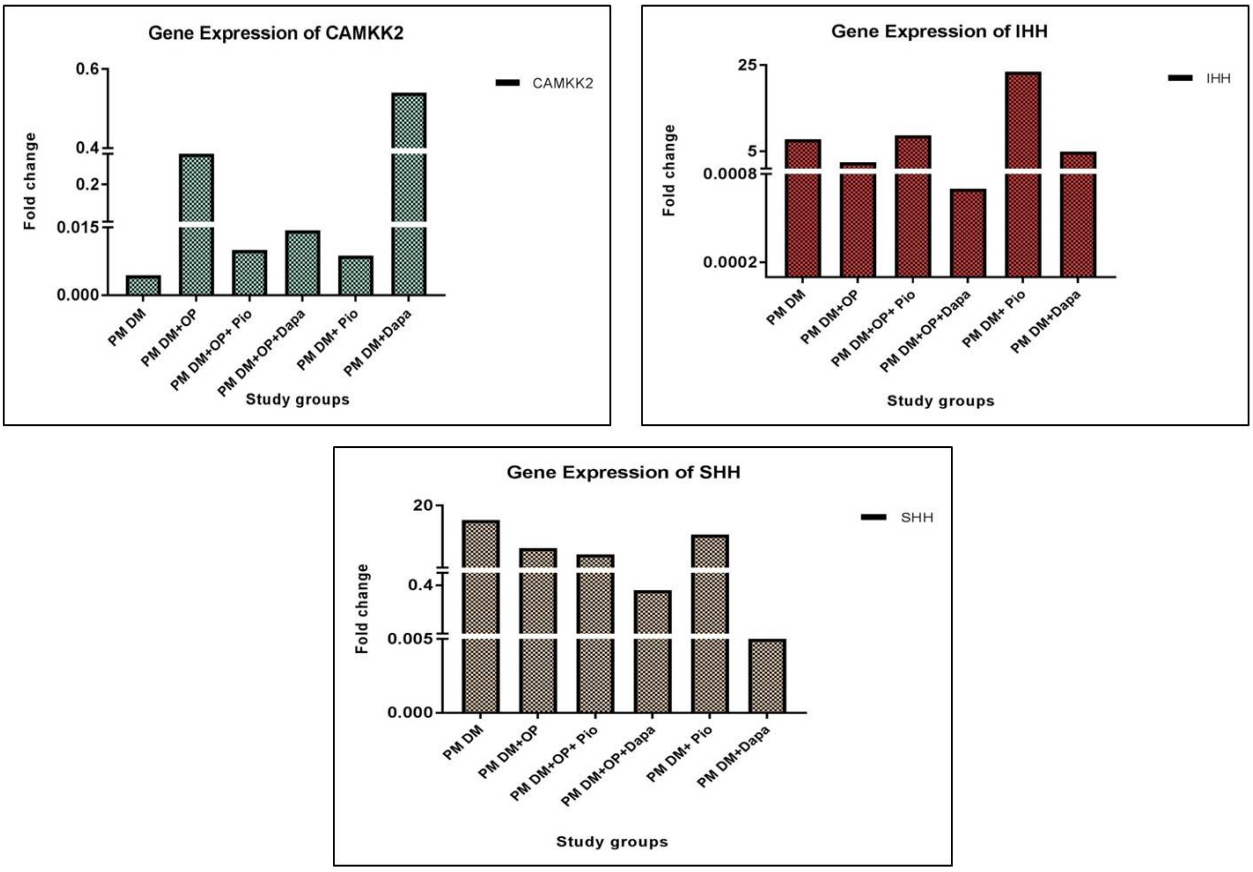
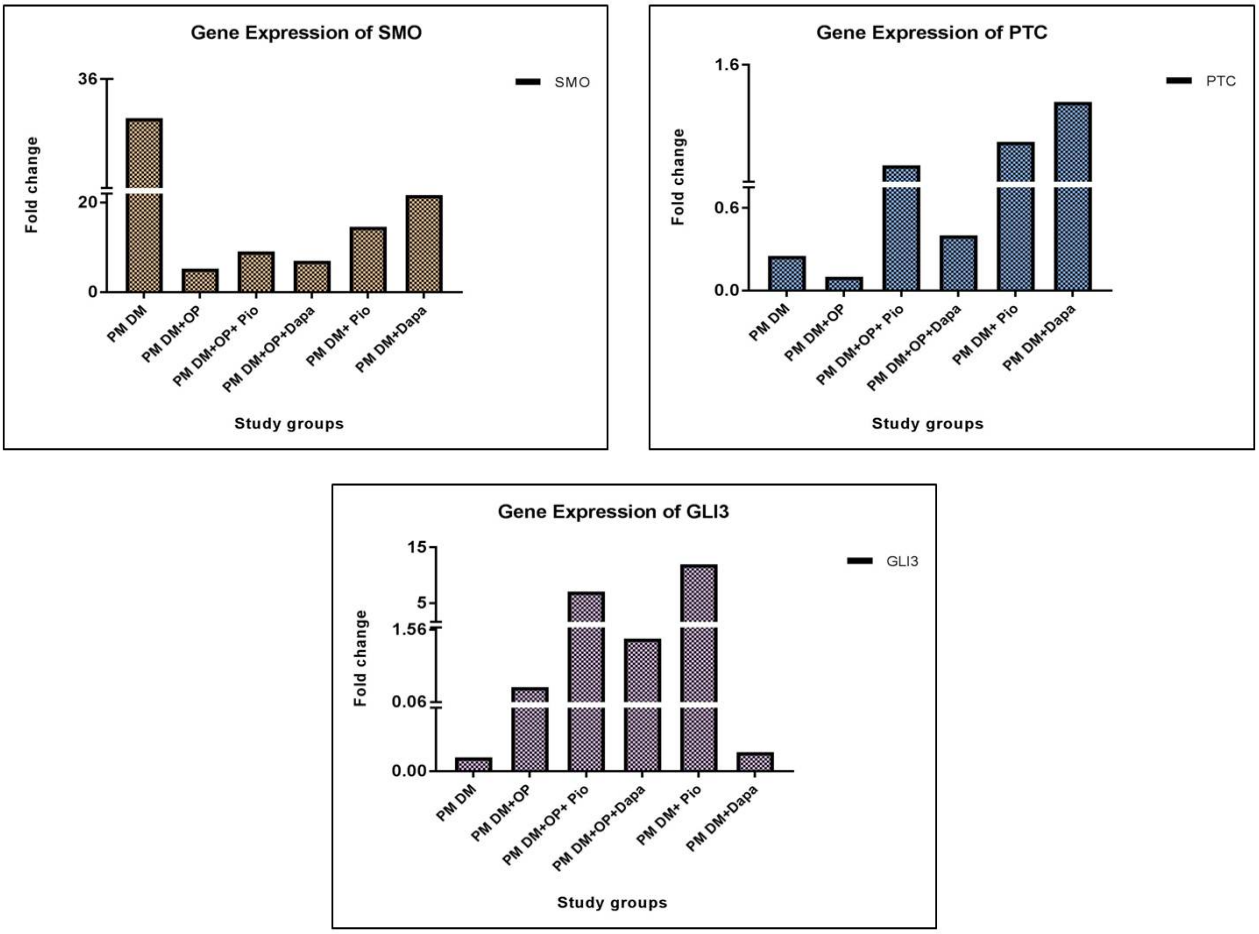


Fig 4: Relative gene expression of CaMKK2, IHH, SHH was determined using q-RT PCR and the fold change ($2^{-\Delta\Delta Ct}$) is presented. The $2^{-\Delta\Delta Ct}$ values were derived with reference to PM healthy control groups. All experiments were repeated as triplicates.

Fig.5 : Relative gene expression of SMO, PTC and GLI3 in the study groups



Fig,5: Relative gene expression of SMO,PTC,GLI3 was determined using q-RT PCR and the fold change ($2^{-\Delta\Delta Ct}$) is presented . $2^{-\Delta\Delta Ct}$ values were derived with reference to PM healthy control groups. All experiments were repeated in triplicates.

1. The results indicate that SMO and PTC gene expressions were several fold lower in the PM DM+ OP group when compared to the other groups. The present study results also reveal a low IHH expression in the PM DM+OP groups reflecting the possibility that CaMKK2 effects bone weakening and enhances bone resorption via its targets, IHH, SMO/ PTC and thereby may sequentially, relatively lower GLI3 mediated transcriptional activity.

2. Both IHH and SHH levels were significantly reduced in the PM DM+dapa treated group, while IHH levels were several fold lower in the PM DM+OP+dapa group of patients followed by the PM DM OP group strongly indicating that CaMKK2 may play a predominant role in the Dapagliflozin treated group via IHH, enabling bone resorptive changes.

3. The PM DM+Pio patient group exhibited a marked elevation in the expression of IHH and GLI3. They also reveal a relatively higher levels of SHH indicating that pioglitazone may exhibit differential osteoporotic effects that are primarily not associated with IHH signaling.

Taken together, it can be suggested that in line with earlier findings (8, 9) PM DM+ OP group of patients may have differential gene expression of CaMKK2 and its associated targets which inhibit/disrupt bone remodeling mechanisms, and favor high bone mineral density, bone repair and formation. Evaluation of CaMKK2 in osteoporotic, postmenopausal diabetic osteoporotic samples along with activation assays would help ascertain the data and enable assessing for CaMKK2 as one of the diagnostic markers for osteoporosis, utilizing blood samples.

CaMKK2, PPAR-γ and PI3K in the Pioglitazone and Dapagliflozone, treated group of patients

Of the several group of drugs that enable superior diabetic control the thiazolidenediones (TZDs) and the sodium-glucose cotransporters (SGLTs) inhibitors stand out. While their antidiabetic potential is well established, prevailing reports also indicate the risk of secondary osteoporosis associated with these class of drugs. The TZD therapeutic drugs Pioglitozone and Rosaglitazone are reported to effect secondary osteoporosis in elderly/postmenopausal women through PPAR-γ mediated mechanisms. Primarily, PPAR-γ mediates bone resorption, inhibits bone formation by interfering with the homeostatic mechanisms associated with bone remodeling{Lecka-Czernik, 2010 #17}.

Fig. 6: Relative gene expression of PPAR-γ and PI3K in the study group patients

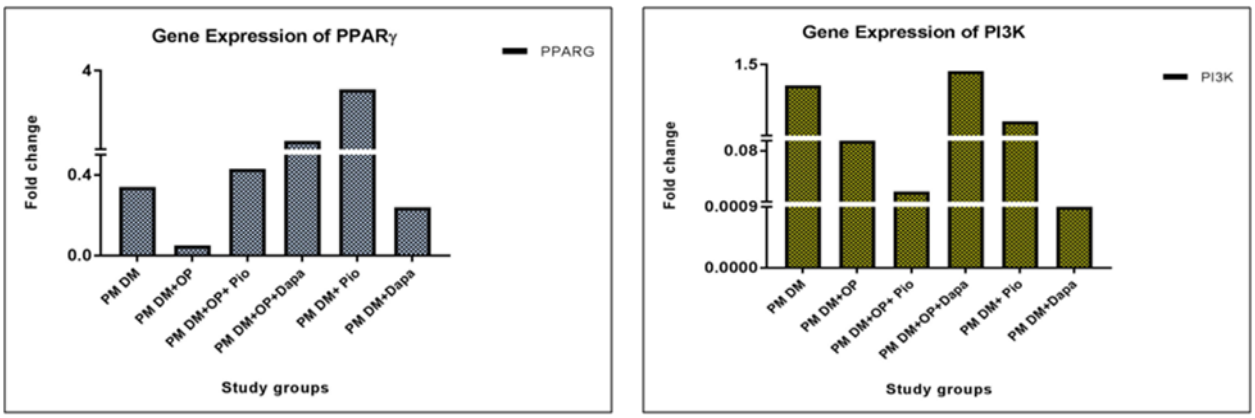


Fig 6: Relative gene expression of PPARG, PI3K was determined using q-RTPCR and the fold change $2^{-\Delta\Delta Ct}$ is presented. $2^{-\Delta\Delta Ct}$ values were derived with reference to PM healthy control groups. All experiments were repeated as triplicates.

1. The results presented in Fig.6 brings forward that as expected PPAR-γ gene expression levels were markedly enhanced in the Pioglitazone treated PM DM patients (PM DM+Pio). Looking across the data, and as observed earlier, the PM DM+Pio group of

patients had a relatively higher expression of IHH, SMO, PTC and GLI3 indicating that the PPAR-γ mediated mechanisms may predominate in these patients. It can further be observed from the figures that patient groups presenting higher levels of CaMKK2 had relatively lower fold expression of PPAR-γ (PM DM+OP, PM DM+Dapa), introducing the possibility that CaMKK2 may inhibit PPAR-γ mediated mechanisms/ PPAR-γ mediated cell specific mechanisms.

2. The PM DM +OP+ Dapa treated group of patients exhibited a relatively higher increase in PPAR-γ expression indicating that PPAR-γ may participate in a resorptive function in the bone.

The renal sodium-glucose cotransporter(s) (SGLTs) inhibitors, in particular the sodium-glucose cotransporter 2 inhibitors, serve as excellent anti-hyperglycemic drugs as they enhance urinary glucose excretion. Therapy with SGLT2 inhibitors has been observed to elevate serum phosphate due to phosphate resorption, cause an increase in PTH and consequently effect bone turnover, density and fracture risk (13). PI3K is a central signaling candidate connected to serum calcium, phosphorous changes and PTH signaling. Very recent evidences have also come out with significant findings that PI3K-Akt pathways are strongly associated stress fracture repair and thereby the osteogenic repair pathway(14). While downstream mechanisms via NFKB (15), have been identified, the mechanism/cross talk mechanisms pertaining to CaMKK2 to is practically unpublished/unexamined. Hence we sought to identify the modulations of PI3K also in the study group patients.

1. <u>It can be observed from the data that PI3K relative gene expression was lower in the Pioglitazone treated group of patients (PM DM+OP+Pio as well as in the Dapagliflozin treated group of patients without osteoporosis (PM DM+Dapa).</u>

2. Further cell specific, promoter specific assays would enable better understanding of the PI3K, PPAR-γ dynamics in the context of postmenopausal diabetic osteoporosis.

<u>Summary</u>

- Relative gene expression studies in blood cells/ samples from postmenopausal osteoporosis patients reveal that CaMKK2 and its target genes may be differentially expressed. Further studies that determine the associated translational/ post translational modifications will ascertain the efficacy of CaMKK2 to serve as a diagnostic, prognostic marker in osteoporosis.
- The relative differences in the gene expression pattern in the Pioglitazone, dapagliflozin treated group of patients strongly indicate that CaMKK2 may participate/intervene in the PPAR-γ, PI3K mediated dynamics and such cross talk mechanisms mediated by CaMKK2 may modulate therapeutic outputs in elderly female patients with diabetes and osteoporosis.
- Activation/phosphorylation studies of CaMKK2 and its downstream signaling candidates is essential to validate the present results and demonstrate the efficacy of CaMKK2 as a diagnostic marker.

References:

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