Diabetic Complications Consortium – Progress Report

Application Title: "Role of NGAL in Regulation of FGF23 in Diabetic Kidney Disease"
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1. Project Accomplishments:

The overall goal of this project is to evaluate the role of NGAL and other inflammatory mediators in regulation of FGF23 in DKD, since in our preliminary results we found NGAL to have very close relationship with FGF23 despite controlling for other factors considered responsible for regulation of FGF23.

Using the funding offered by DiaComp Consortium, our cell culture experiments have demonstrated direct stimulatory effect of TNF-α and IL-6 on Osteocytes to produce FGF23, in addition to NGAL. The cross-sectional and prospective analyses of blood sample from 51 diabetic nephropathy patients show the independent persistent relationship between FGF23 and NGAL, although this part of analysis is limited due to slow recruitment of the patients for the ongoing parent study.

In summary, the available data strengthens the role of NGAL in the regulation of FGF23 and based on these results, we have designed animal studies exploring the role of inhibition of NGAL in diabetic kidney disease using NGAL knockout mice.

2. Specific Aims:

Specific Aim 1. To investigate both cross-sectionally and prospectively the relationship of FGF23 and NGAL in patients with moderate and advanced diabetic nephropathy.

Methods: By May 2014, 52 patients had completed the parent study and we analyzed their blood samples for FGF23 and NGAL on two occasions with 3-month interval. In addition to above, we had assessment of CBC, and basic metabolic panel in these patients. Analysis of bone parameters, iron metabolism and dietary intake of phosphorus is pending to date, waiting for completion of the study.

Results:

Table 1: Demographic and biochemical characteristics of study population

Parameters	At Baseline	3-month Interval
Age (y)	61.5 ± 7.5	
Sex (% male)	85	
Race (%)	71- Hispanic white	
BMI	35.3 ± 8.84	
eGFR (ml/mim)	32.82 ± 12.4	32.2 ± 15.2
WBC (x1000/ml)	7.38 ± 1.8	7.35 ± 2.06
Hemoglobin (Hgb) (g/dl)	11.75 ± 1.7	11.61 ± 1.6
Phosphorus (mg/dl)	4 ± 0.8	4.17 ± 0.89
Calcium (mg/dl)	8.8 ± 0.4	8.89 ± 0.41
Albumin (g/dl)	3.38 ± 0.5	3.38 ± 0.4
FGF23 (RU/ml)	62.75 (34.6, 131.5)	78.8 (45.8, 123.8)
NGAL (ng/ml)	359 (262.5, 533.4)	404.7 (269.2, 501.4)

Table 2: Univariate relationship of demographical and biochemical parameters from the baseline period

	Age	Sex	Race	BMI	eGFR	WBC	Hgb	Albumin	Phos	Calcium	Hgb A1C	Log_{10} FGF23	Log_{10} NGAL
Age	1												
Sex	0.03	1											
Race	0.2	0.1	1										
BMI	-0.2	-0.1	0.05	1									
eGFR	-0.1	0.3*	-0.1	0.0	1								
WBC	-0.2	-0.3	-0.3	0.3	-0.1	1							
Hemoglobin	0.13	0.3*	0.1	0.1	0.5*	0.05	1						
Albumin	0.2	0.2	-0.3*	0.0	0.3	-0.1	0.5*	1					
Phosphorus	-0.1	0.4*	-0.1	0.2	0.6*	0.2	0.4*	0.0	1				
Calcium	0.24	-0.1	0.1	0.1	0.2	-0.04	0.5*	0.6*	-0.1	1			
HgbA1C	-0.1	0.2	0.2	0.1	-0.02	0.1	0.1	0.0	-0.14	0.04	1		
Log_{10}FGF23	-0.1	-0.3	-0.1	0.22	-0.66*	0.43*	-0.3*	-0.18	0.55*	0.2	0.05	1	
Log_{10}NGAL	-0.2	-0.4*	-0.1	0.18	-0.71*	0.5*	-0.4*	-0.28*	0.58*	0.3	0.02	0.7*	1

Table 3: Linear regression analysis with Log_{10}FGF23 as dependent variable (model 1)				
	Beta	**SE**	**t**	**p**
Constant		0.93	0.55	0.58
Age	-0.09	0.005	-0.81	ns
eGFR	-0.337	0.005	-2.03	0.048
Phosphorus	0.157	0.06	1.18	ns
Albumin	0.05	0.09	0.4	ns
Hgb	0.015	0.029	0.11	ns
Log_{10}NGAL	0.36	0.25	2.27	0.028

Table 4: Linear regression analysis with Log_{10}FGF23 as dependent variable (model 1 + WBC)				
	Beta	**SE**	**t**	**p**
Constant		0.94	1.53	0.13
Age	-0.09	0.005	-0.87	ns
eGFR	-0.50	0.005	-3.0	0.005
Phosphorus	0.136	0.06	1.09	ns
Albumin	0.09	0.09	0.79	ns
Hgb	-0.06	0.03	-0.44	ns
WBC	0.33	0.026	2.61	0.013
Log_{10}NGAL	0.07	0.29	0.39	ns

In an additional regression model using NGAL as the dependent variable, eGFR, plasma hemoglobin, albumin, phosphorus and absolute neutrophil count explained 75% of NGAL variation ($p<0.001$), with partial F-test analysis showing significance for only eGFR ($\beta=-0.47$, $p<0.001$) and ANC ($\beta=0.40$, $p<0.001$).

The same analyses were performed on blood sample collected at 3-month period and similar results were obtained, assuring the validity of the results.

Conclusion: The results of our study suggest

1. NGAL remains a significant predictor of FGF23 in diabetic kidney patients. Since NGAL is a product of white blood cells, this relationship is lost when WBC is included in the model, also suggesting that peripheral leucocytes are a major source of NGAL production in CKD patients.
2. Elevated serum NGAL is a function of both decreased kidney function and circulating neutrophils in CKD patients.

Progress: Since May 2014 to till date, we have another 30 completers, total completers = 82. We anticipate parent study to complete by middle of next year. Once the study is completed, the final analysis of relationship between NGAL and FGF23, while controlling for all the available confounding factors (parameters of bone and iron metabolism and dietary phosphorus) will be conducted. Meanwhile, the available data supports our current hypothesis.

Specific Aim 2. *To investigate the effect and mechanism of action of NGAL on FGF23 production in vitro.*

Methods: For this aim, osteocytes were incubated with varying concentrations of NGAL, TNF-α and IL-6.

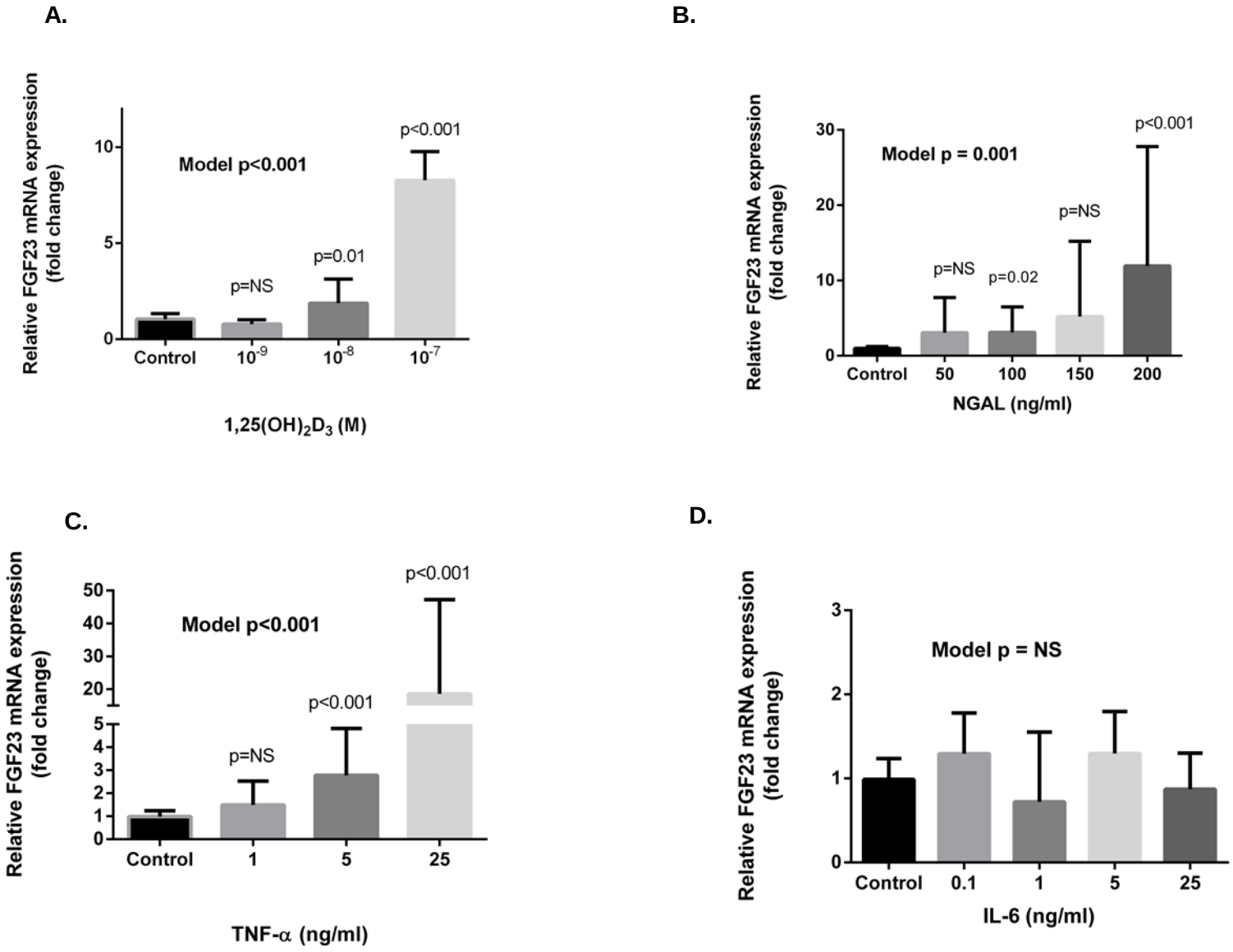

Figure 1: Real-time PCR analysis of FGF-23 mRNA from IDG-SW3 osteocytes in culture. FGF23 mRNA abundance normalized for RPL13A housekeeping gene and expressed as fold-change (mean±SD) relative to control, after incubation of IDG-SW3 osteocytes with A) 10^{-9} to 10^{-7}M $1,25(OH)_2D_3$ [average n=7 for each dose]; B) 50-200 ng/ml NGAL [average n=15 for each dose]; C) TNF-α 1-25 ng/ml [average n=16 for each dose]; D) IL-6 0.1-25 ng/ml [average n=9 for each dose]; or vehicle [n=27] for 24 hours. P value is derived for the whole model and each individual dose compared to the control group using univariate analysis of variance with contrast.

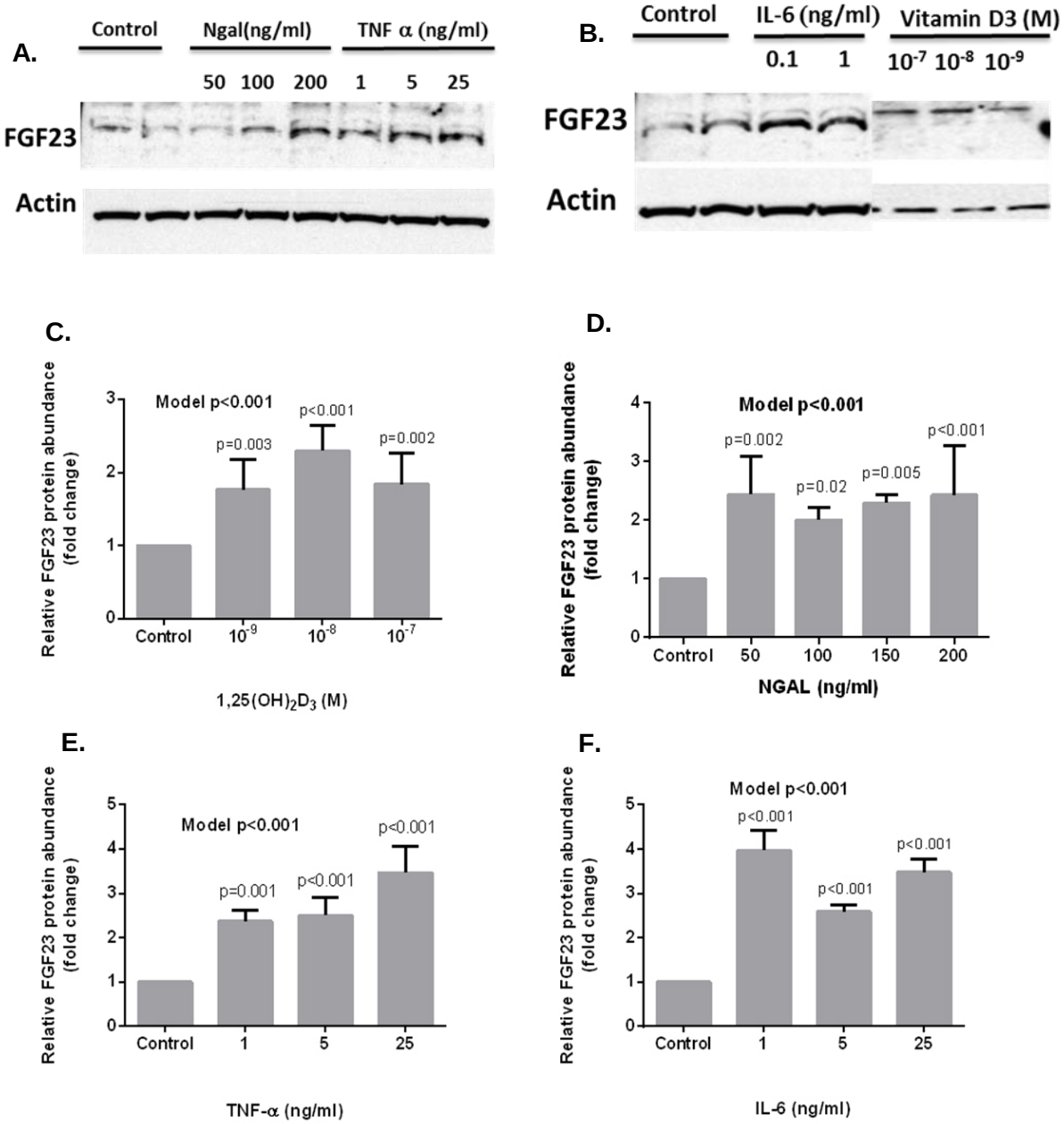

Figure 2. SDS-PAGE analysis of FGF-23 protein in the cytosol of IDG-SW3 osteocytes. *Panels A and B:* representative examples of FGF23 and actin protein bands after 48-hour incubation of the cultures with 50-200 ng/ml NGAL or 1-25 ng/ml TNF-α (Panel A), or with 0.1-25 ng/ml IL-6 or 10^{-9}-10^{-7}M $1,25(OH)_2D_3$ or vehicle (Panel B). *Panels C through F*: average FGF23 band density after incubation with $1,25(OH)_2D_3$ (Panel C, n=3/dose), NGAL (Panel D, n=4/dose), TNF-α (Panel E, n=4/dose) and IL-6 (Panel F, n=2/dose). FGF23 band density is normalized for that of actin and it is shown as mean fold-change relative to control ± SD. Statistical analysis consisted of GLM analysis with FGF23 as dependent variable (Model *p* value) and contrast between each treatment dose and control (individual dose *p* values).

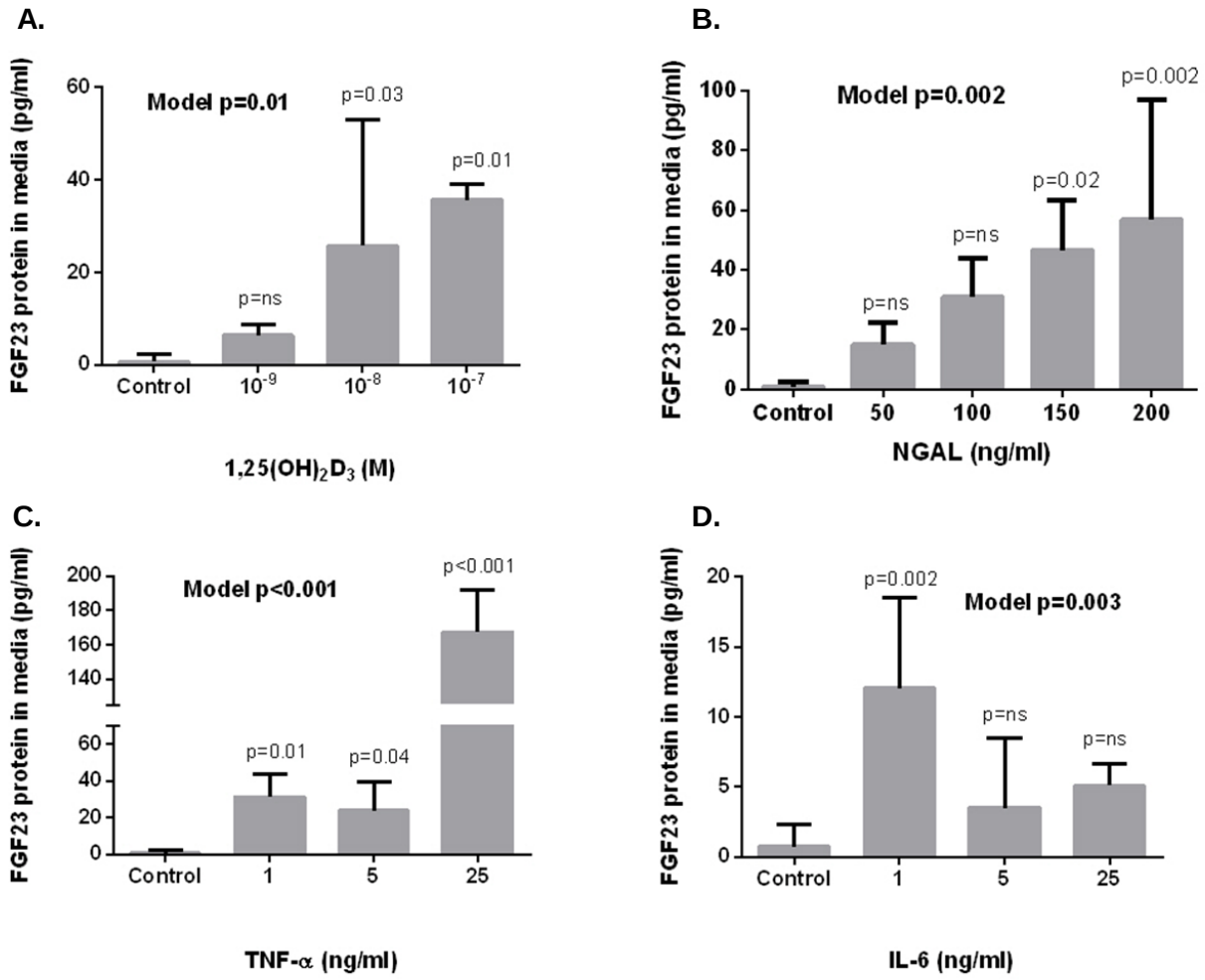

Figure 3. ELISA of FGF-23 protein in conditioned medium of IDG-SW3 osteocyte cultures. FGF23 concentration measured after 48-hour incubation with 10^{-9}-10^{-7}M $1,25(OH)_2D_3$ (Panel A), 50-200 ng/ml NGAL (Panel B), 1-25 ng/ml TNF-α (Panel C) and 1-25 IL-6 ng/ml (Panel D). Each bar represents the average (mean ± SD) of 2-3 determinations. Statistical analysis consisted of GLM analysis with FGF23 as dependent variable (Model *p* value) and contrast between each treatment dose and control (individual dose *p* values).

Conclusion: Both TNF-α and NGAL stimulate the increased synthesis of FGF23 mRNA and protein from osteocytes. IL-6 increases FGF23 protein synthesis, but not mRNA suggestive post-translational modification.

Progress: To confirm the role of NGAL on FGF23 stimulation and to evaluate the mechanism, experiments are underway utilizing SiRNA for megalin and 24P3 receptor, the two known receptors mediating the action of NGAL.

3. **Publications**:

1. Inflammatory cytokines including NGAL associate with elevated FGF23 levels in chronic kidney disease patients and stimulate FGF23 synthesis in osteocytes. Original article. Am J Physiol Renal Physiol (Under resubmission process)
2. Elevated neutrophil gelatinase-associated lipocalin (NGAL) level in chronic kidney disease (CKD) is function of both reduced glomerular filtration rate (GFR) and circulating neutrophil count. Abstract to be presented at American Society of Nephrology Annual Meeting at Philadelphia in Nov. 2014
3. Neutrophil Gelatinase-Associated Lipocalin and Inflammatory Mediators Stimulate Fibroblast Growth Factor-23 Production in in vitro Osteocytes Culture. Abstract to be presented at American Society of Nephrology Annual Meeting at Philadelphia in Nov. 2014

4. **Grants**:

    1. Local Institution of Integrated Medicine and Science –Pilot grant, direct cost $50,000 for one year.
    2. I plan to apply for K23 early next year.