Supplementary Information

TonEBP mediates hyperglycemia-induced inflammation, and vascular and renal injury

Soo Youn Choi[1,#], Sun Woo Lim[2,#], Shabnam Salimi[3], Eun Jin Yoo[1], Whaseon Lee-Kwon[1], Hwan Hee Lee[1], Jun Ho Lee[1], Braxton D. Mitchell[3,4], Satoru Sanada[3], Afshin Parsa[3,5,*], Hyug Moo Kwon[1,*]

[1]School of Life Sciences, UNIST, Korea; [2]Transplantation Research Center, Catholic University of Korea; [3]Department of Medicine, University of Maryland Baltimore, USA; [4]Geriatrics Research and Education Clinical Center, Baltimore Veterans Administration Medical Center, USA: [5] Department of Medicine, Division of Nephrology, Baltimore Veterans Administration Medical Center, Baltimore, USA.

[#] These authors contributed equally to the study.

* Address correspondence to HMK at hmkwon@unist.ac.kr or AP at aparsa@medicine.umaryland.edu.

Supplementary Results

Mouse model of DN

While most mice not develop many features of DN, mouse lines with endothelial dysfunction, endothelial nitric oxide synthase-deficient (*eNOS*$^{-/-}$) lines, display most features of human DN except for renal insufficiency[1-4]. We decided to adopt this system to test the effects of TonEBP haplodeficiency on the development of DN. For this, we bred the TonEBP haplo-deficient animals onto the *eNOS*$^{-/-}$ line. Type 1 diabetes was induced as described in Fig 1 and the animals were analyzed 7 weeks later. Supplementary Table 1 summarizes physiological parameters of *TonEBP*$^{+/\Delta}$ mice and their *TonEBP*$^{+/+}$ littermates on the *eNOS*$^{-/-}$ and *eNOS*$^{+/+}$ backgrounds. All the parameters were comparable between the *TonEBP*$^{+/\Delta}$ animals and their *TonEBP*$^{+/+}$ littermates, except that renal hypotrophy indicated by reduced ratio of kidney weight over body weight lessened in the *TonEBP*$^{+/\Delta}$ animals on the *eNOS*$^{-/-}$ background. Reduced renal TonEBP expression in the *TonEBP*$^{+/\Delta}$ animals were confirmed on both the *eNOS*$^{-/-}$ and *eNOS*$^{+/+}$ backgrounds (Fig 3a, b).

Association of TonEBP polymorphisms with inflammatory, vascular and renal function markers in humans

In view of our primary animal TonEBP haplo-deficiency findings as noted above, and previously published associations of TonEBP expression and/or gene variants with inflammation[5,6], rheumatoid arthritis[7,8], atherosclerosis[9], and DN in humans[10] and in animals (see above), we hypothesized that genetic variation in TonEBP would be associated with subclinical inflammatory, vascular and renal phenotypes. To test this hypothesis, we performed a look-up of genetic association results carried out in 878 participants from the HAPI Heart Study[11], a study of the genetic determinants of cardiometabolic health carried out in the Old Order Amish founder population of Lancaster, County, PA from 2002 - 2006. HAPI Heart Study

participants were 20 years of age or older, relatively healthy with a homogenous genetic and environmental background population, and free of any medications at the time of study. Characteristics of study participants are summarized in Supplementary Table 2.

Phenotypes measured in the HAPI Heart Study and relevant to this report related to inflammation and renal function included: serum IL-1β an inflammatory cytokine, homocysteine, matrix metalloprotease-1 (MMP-1), matrix metalloprotease-9 (MMP-9), C-reactive protein (CRP), monocyte count and white blood cell count (WBC) as markers of systemic inflammation[12,13]. Renal function phenotypes included: albuminuria and estimated glomerular filtration rate (eGFR). Resting protocol based systolic and diastolic blood pressure (SBP and DBP) measures were also obtained while participants were off any anti-hypertensive medications.

All HAPI Heart Study participants underwent whole genome sequencing as part of the NHLBI-sponsored Trans-Omics in Precision Medicine (TOPMed) program. For this report we considered all sequenced variants within the TonEBP gene plus variants ± 2 kb away with a minor allele frequency (MAF) of 3%. From these 320 SNPs, using Haploview, we identified 16 haplotype blocks[14]. We then identified all single haplotype tagging SNPs (Supplementary Figure 1) and looked at the association between the identified SNPs and our phenotypes.

For each phenotype or trait, we identified the top associated signals with a P value < 0.05 (Table 1). We defined statistical significance threshold based on a Bonferroni correction for the number of haplotype blocks (p value < 0.05/16 = 0.003). Considering that our animal model demonstrated the effect of TonEBP haplo-deficiency on these phenotypes, increasing the a-priori probability of true associations, we also identified SNP based associations with p values < 0.05 but > 0.003 as suggestive. For our inflammatory phenotypes, we found significant associations between rs72783114 with serum MMP-1 (β = -0.31, p = 0.0003) and suggestive association with serum WBC (β = 0.06, p = 0.04). We also found rs564919090 to be significantly associated with serum MMP-1 (β = 0.28, p = 0.001), independently of rs72783114

(Table 1). Given our previous findings of higher TonEBP expression in monocytes from individuals with DN[10], we also looked at the association with absolute monocyte values, which were available in a different group of 473 healthy Amish and found a significant association between rs118095741 with absolute monocyte count (β = 47.3, p = 0.002). We also found rs74956396 to be suggestively associated with serum IL-1β (β = 0.52, p = 0.009) and homocysteine (β = -0.61, p = 0.008), while rs244416 was independently also suggestively associated with IL-1β (β = -0.22, p = 0.009). For our blood pressure phenotypes, we found rs2287970 to be significantly associated with DBP (β = 1.4, p = 0.003) and suggestively with SBP (β = 1.65, p = 0.04). Lastly, for our renal phenotypes, we found a significant association between rs17297179 with eGFR (β = 6.3, p = 0.003) and suggestive association between rs17232663 with albuminuria (β = 0.36, p = 0.008). Given that blood pressure (BP) can affect renal function and vice versa, we secondarily adjusted for BP in our eGFR and albuminuria outcomes, as well as adjusted for eGFR with our BP outcomes, which did not meaningfully alter the results. Lastly, the noted associations with our inflammatory markers were also independent of eGFR and BP.

We did not find an association with CRP. This might be accounted for by the modest number of individuals in our study, as in a previous secondary analysis of a uric acid meta-GWAS in which we participated, a modest association between the rs7193778 and CRP was noted, though other TonEBP SNPs were not tested[43]. We also did not find an association between our selected TonEBP variants and MMP-9.

Box plot graphs showing the mean values and distribution for each of our significantly associated measures stratified by genotype can be seen in Supplementary Fig 2. Locus zoom plots showing location of significantly associated SNPs with each phenotype can be seen in Supplementary Fig 3.

We also explored the functional annotation for our top identified SNPs which are summarized in Supplementary Table 3. Of these eight SNPs of interest, five of them had

enhancer activity defined with H3K27AC marker in various tissues, but rs2287970 and rs17297179 were also notable as active enhancer with DNase hypersensitivity, which suggests them to be functionally active. Variation in rs2287970 also results in change in various motifs with zinc-finger domain such as WTN1 and Znf143 (Supplementary Table 3).

Supplementary Table 1. Physiological parameters of experimental animals.

Parameter	eNOS (+/+)				eNOS (-/-)			
	TonEBP (+/+)		TonEBP (+/Δ)		TonEBP (+/+)		TonEBP (+/Δ)	
	VH	STZ	VH	STZ	VH	STZ	VH	STZ
Body weight (g)	28.9 ± 0.6	24.1 ± 0.8	28.9 ± 0.9	22.8 ± 0.7	24.4 ± 0.7	20.7 ± 0.8	24.3 ± 0.6	20.8 ± 0.6
Kidney/Body weight (mg/g)	14.3 ± 0.6	17.1 ± 0.9	13.2 ± 0.4	16.4 ± 0.9	7.7 ± 0.4	12.9 ± 0.4	8.6 ± 0.6	15.6 ± 0.4$^{\#}$
Blood glucose (mg/dL)	163 ± 12	453 ± 31	150 ± 18	482 ± 34	198 ± 12	560 ± 31	177 ± 22	577 ± 13
Spot urine osmolality (mmoL/kg)	1894 ± 130	787 ± 110	1627 ± 173	813 ± 31	1205 ± 107	541 ± 36	1057 ± 109	545 ± 25
Blood urea nitrogen (mg/dL)	11.7 ± 0.8	20.0 ± 1.9	12.6 ± 0.7	17.9 ± 1.1	28.4 ± 2.3	25.3 ± 2.2	30.5 ± 1.3	32.3 ± 1.9
FE_{Na} (%)	0.47 ± 0.08	0.85 ± 0.24	1.16 ± 0.28	2.38 ± 0.72	0.64 ± 0.10	1.17 ± 0.17	0.41 ± 0.05	1.15 ± 0.21

Mice were bred to generate littermates of *TonEBP*$^{+/+}$ or *TonEBP*$^{+/\Delta}$ animals on *eNOS*$^{+/+}$ or *eNOS*$^{-/-}$ background as indicated. Animals were injected with vehicle (VH) or streptozotocin (STZ) to induce diabetes as described in Fig 1. 7 weeks later, body weight and kidney weight were measured; serum and urine samples were collected to determine blood glucose, urine osmolality, blood urea nitrogen, and fractional excretion of sodium (FE_{Na}). Mean ± SE, n = 8. $^{\#}p< 0.05$ compared to *TonEBP*$^{+/+}$, *eNOS*$^{-/-}$.

Supplementary Table 2. Clinical characteristics (mean ± SD; median (IQR)) of the 868 Old Order Amish enrolled in the HAPI Heart Study, Lancaster County, Pennsylvania

Characteristic	Men (*n*=460)	Women (*n*=408)
Age (years)	42.2 ± 13.6	45.4 ± 14.2
BMI (kg/m^2)	25.6 ± 3.2	27.8 ± 5.5
Total cholesterol (mg/dl)	202.5 ± 44.3	215.7 ± 49.0
Triglycerides (mg/dl)	63.9 ± 1.7	73.8 ± 45.4
SBP (mm Hg)	121.5 ± 12.6	121.4 ± 16.9
DBP (mm Hg)	77.6 ± 8.8	75.8 ± 8.4
Diabetes (%)	0.9	1.0
Current smokers (%)[a]	20.0	0.0
Lipid-lowering meds (%)[b]	1.0	1.0
Antihypertensive meds (%)[b]	0.2	0.3
IL-1β (pg/mL)	4.2±10.1 0.78(0.78-1.38)	4.6±11.1 0.78(0.78-1.22)
MMP-1(ng/ml)	3.6±2.4 2.9(1.8-4.7)	3.9±2.5 3.2(2.0-5.5)
MMP-9 (ng/ml)	561±343 474(315-725)	511.6±340.8 413(263-673)
CRP (pg/mL)	2.3±6.3 0.8(0.3-1.7)	1.8±2.3 1.0(0.6-2.4)
eGFR (ml/min/1.73m^2)	101.0±17.7	90.0±17.4

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; HAPI, Hereditary and Phenotype Intervention; meds, medication; SBP, systolic blood pressure.
[a]Indicates use of cigarettes, cigars, and pipes. [b]Medication use assessed at the time of recruitment, before participants were asked to discontinue use, per our study protocol. IQR: interquartile range,MMP-1: metaloproteinase-1, CRP: C-reactive protein, eGFR: estimated glomerular filteration rate (ml/min/1.73m^2).

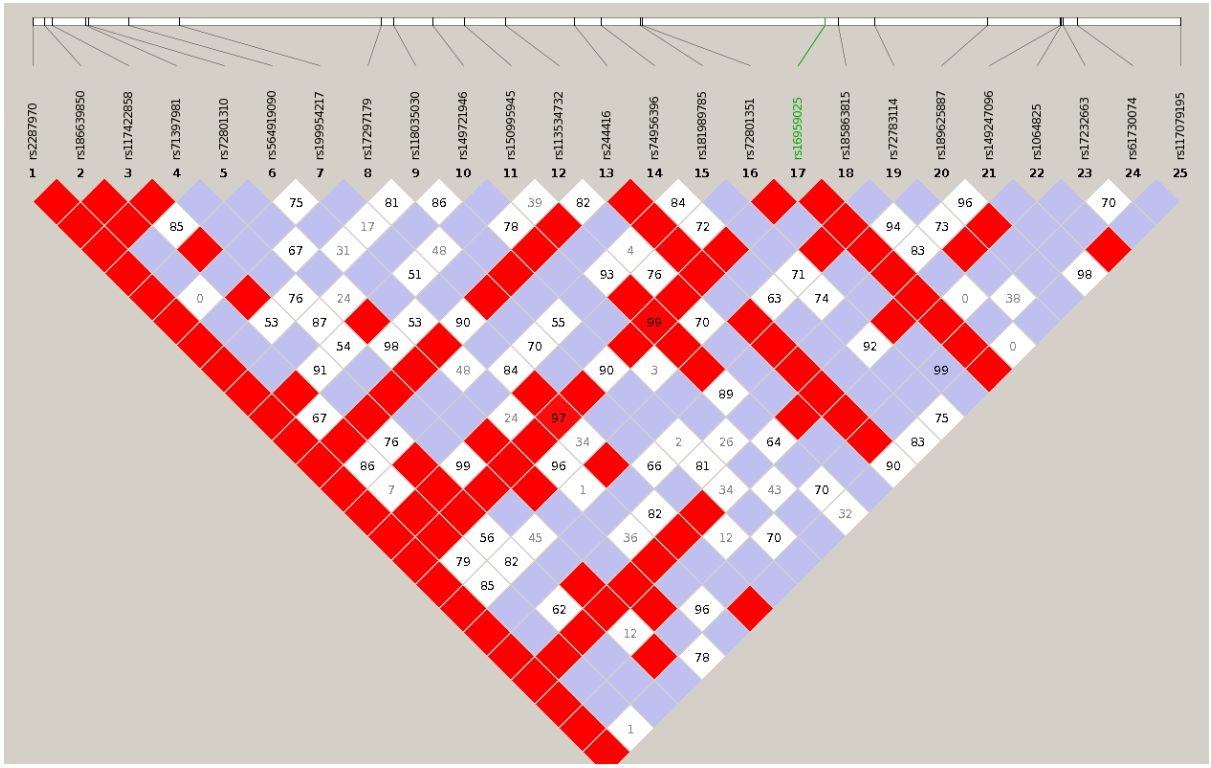
Supplementary Table 3. *In silico* functional annotation of candidate TonEBP SNPs

	Active Enhancer H3K27AC H3K4me1	**DNAase I hyper sensitivity**	**Conservation Phylop Mean(SD)**	**Motif change**	**GTEX**	**DNase and active enhancer**
rs2287970	47 tissues	47 tissues	0.29 (1.07)	15 including WT1, Znf143	WWP2 NPIPB14P	In 40 tissues
rs72783114	10 tissues	-	0.33 (0.9)	AIRE, Fox, HDA	-	-
rs564919090			0.13 (0.7)			
rs118095741	7 tissues		0.09 (0.55)	SEF1	WWP2-PDXDC2P	-
rs74956396	-	-	0.27 (0.7)	-	CTD, NQ1	-
rs244416	-	-	-0.001 (0.6)	Crx	CLEC18C CLEC18A PDXDC2P	
rs149721946	15 tissue	-	-0.04 (0.9)	HDAC2, Irf, TATA, Zfp105	-	-
rs17232663	-	-	-0.11 (0.98)	Ets,SIX5	CLEC18A	-
rs17297179	Primary T helper cell	Primary T helper cell	0.40 (0.8)	BCL, NFKB, STAT, P300, PRDM1, Pou2f2, HMG-IY, Irf, Maf, Nkx3, GATA		Primary T helper cell

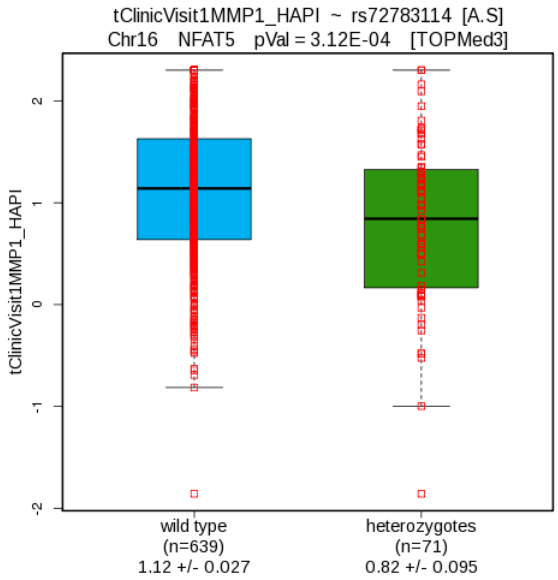
Supplementary Table 4. Primer pairs used for quantitative RT-PCR

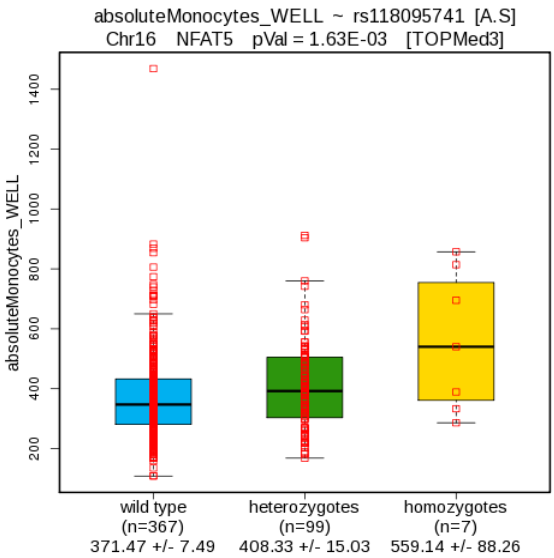
Genes	NCBI ref seq	Forward	Reverse
Mouse TonEBP	NM_133957	AAGCAGCCACCACCAAACATGA	AAATTGCATGGGCTGCTGCT
Mouse TNFa	NM_013693.2	TGGGACAGTGACCTGGACTGT	TTCGGAAAGCCCATTTGAGT
Mouse TLR4	NM_021297.2	TGCTGCCAACATCATCCAGGAA	AGGCGATACAATTCCACCTGCT
Mouse iNOS	NM_010927	TATGCTGTGTTTGGCCTTGGCT	TGTGGCTCCCATGTTGCATT
Mouse COX-2	NM_011198.3	TGCTGTACAAGCAGTGGCAA	AGGGCTTTCAATTCTGCAGCCA
Mouse CypA	NM_008907.1	CAGCCATGGTCAACCCCACCG	CTGCTGTCTTTGGAACTTTGTCTG
Mouse F4/80	NM_010130.4	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Mouse IL-6	NM_031168.1	ATCCAGTTGCCTTCTTGGGACTGA	TAAGCCTCCGACTTGTGAAGTGGT
Mouse MCP-1	NM_011333.3	AACTGCATCTGCCCTAAGGT	AGTGCTTGAGGTGGTTGTGGAA
Mouse IP-10	NM_021274.2	CCAAGTGCTGCCGTCATTTTC	GGCTCGCAGGGATGATTTCAA
Mouse IL-8	NM_011339.2	GACAGGCAGTGATGCCTAAA	GACTAACGCGGGAATAGAGTATAAG
Mouse IL-1β	NM_008361.3	AGGGCTGCTTCCAAACCTTTGAC	ATACTGCCTGCCTGAAGCTCTTGT
Mouse RANTES	NM_013653.3	ATATGGCTCGGACACCACTC	CCCACTTCTTCTCTGGGTTG
Mouse IL-18	NM_008360.1	CAGCCTGTGTTCGAGGATATG	TCACAGCCAGTCCTCTTACT
Mouse IFN-γ	NM_008337.3	TCAAGTGGCATAGATGTGGAAGAA	TGGCTCTGCAGGATTTTCATG

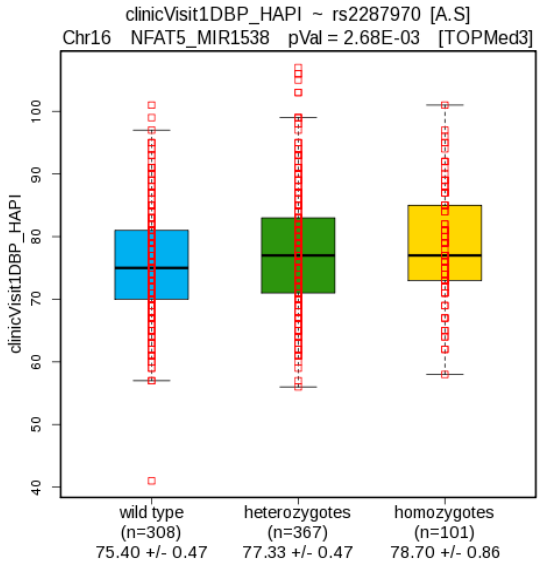
Supplementary Fig 1. D'based haploview for all haplotype tagged SNPs across *TonEBP*.

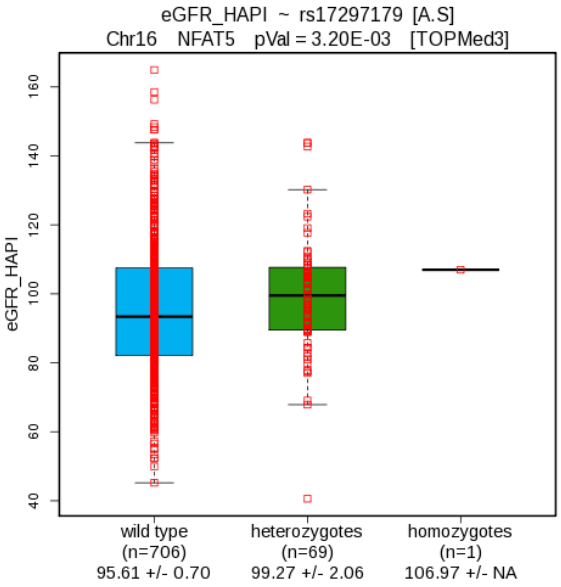


Supplementary Fig 2. Box plot graphs showing the mean values and distribution of phenotypes (MMP1, Monocytes, DBP, and eGFR; see Table 1) across genotypes.



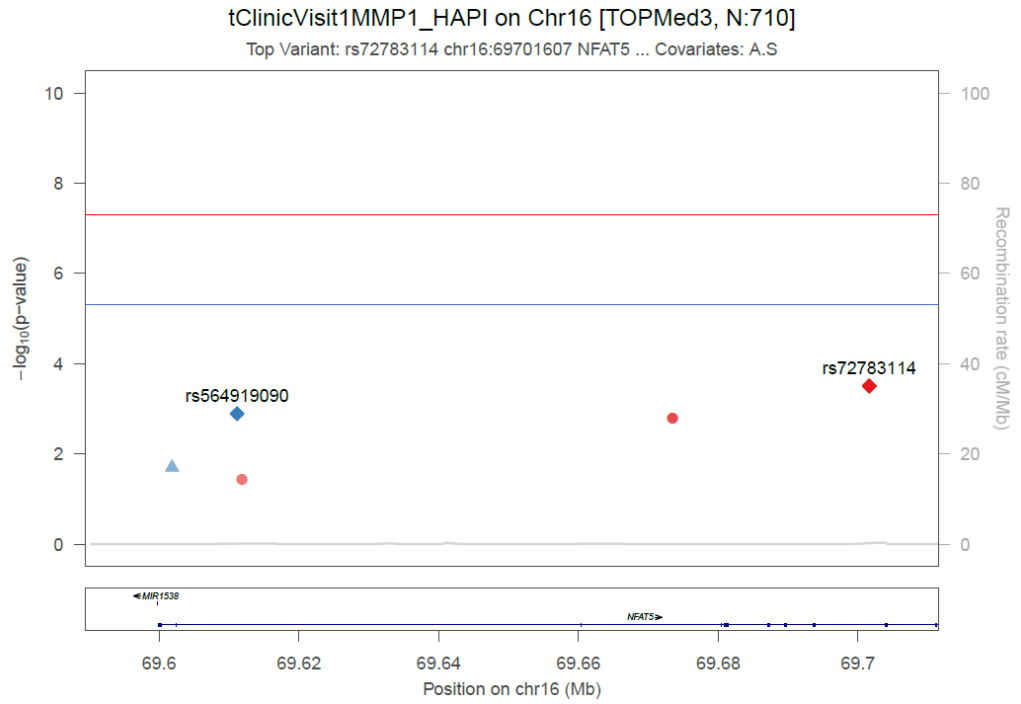


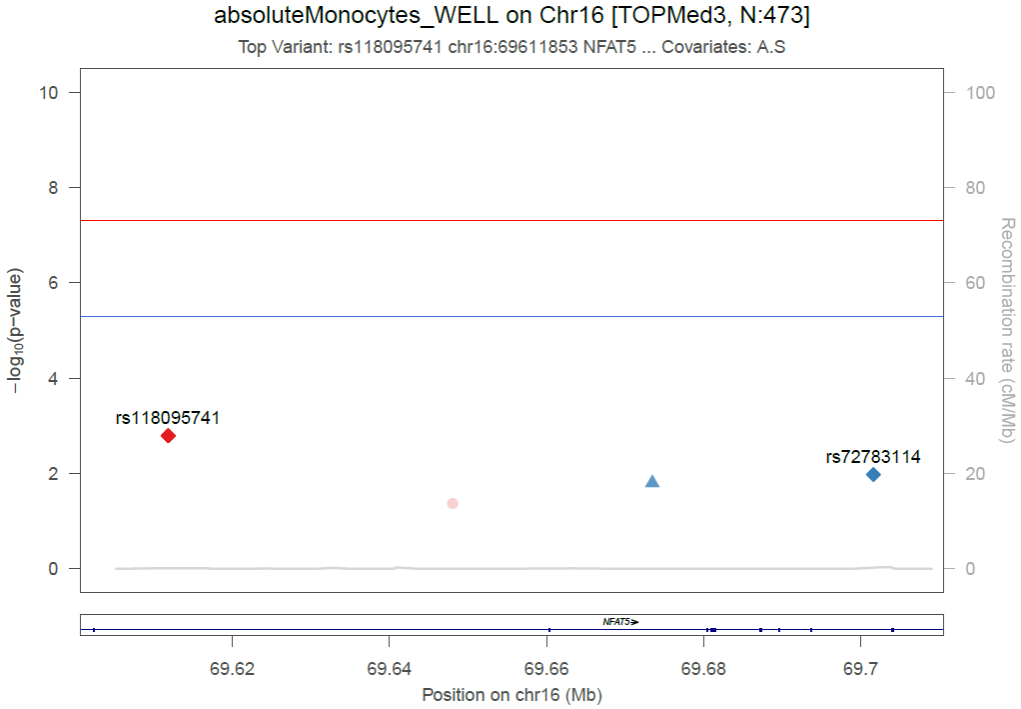


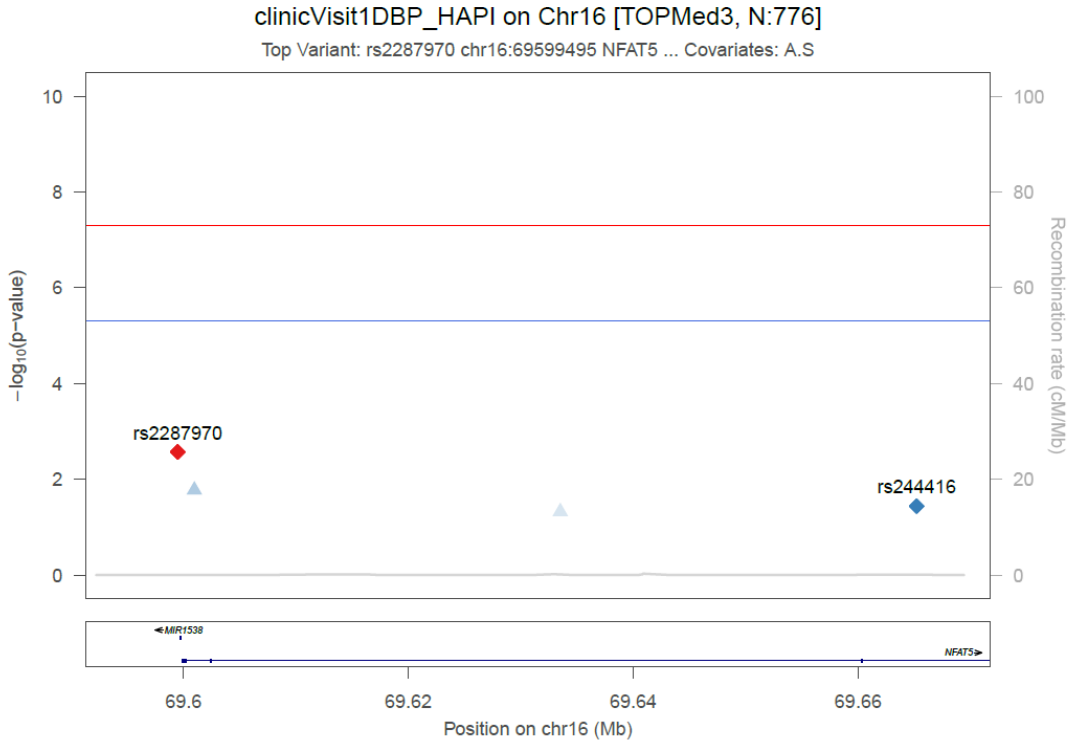


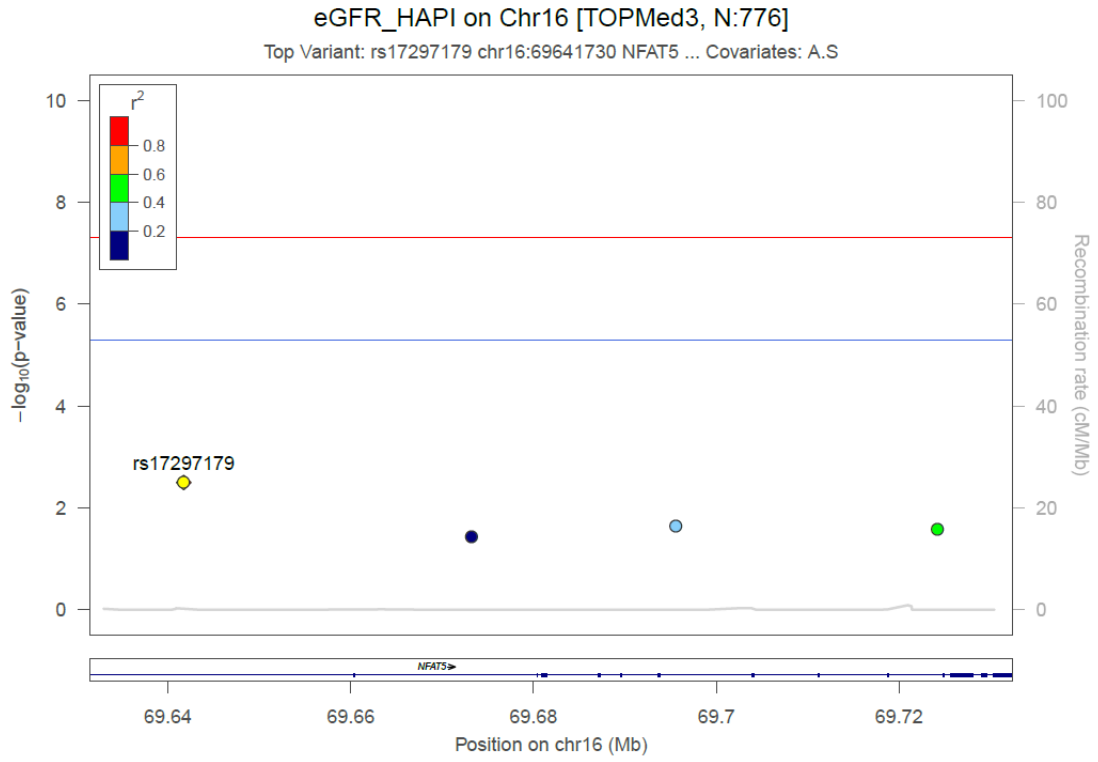
t = log transformed variable

Supplementary Figure 3. Locus zoom plots showing location of all SNPs associated significantly with the phenotypes shown in Supplementary Figure 2









t = log transformed variable

Supplementary References

1. Nakagawa T, Sato W, Glushakova O, Heinig M, Clarke T, Campbell-Thompson M, Yuzawa Y, Atkinson MA, Johnson RJ, Croker B. (2007) Diabetic endothelial nitric oxide synthase knockout mice develop advanced diabetic nephropathy. *J Am Soc Nephrol* 18(2):539–550.
2. Kanetsuna Y, Takahashi K, Nagata M, Gannon MA, Breyer MD, Harris RC, Takahashi T. (2007) Deficiency of endothelial nitric-oxide synthase confers susceptibility to diabetic nephropathy in nephropathy-resistant inbred mice. *Am J Pathol* 170(5):1473-1484.
3. Zhao HJ, Wang S, Cheng H, Zhang MZ, Takahashi T, Fogo AB, Breyer MD, Harris RC. (2006) Endothelial nitric oxide synthase deficiency produces accelerated nephropathy in diabetic mice. *J Am Soc Nephrol* 17(10):2664–2669.
4. Mohan S, Reddick RL, Musi N, Horn DA, Yan B, Prihoda TJ, Natarajan M, Abboud-Werner SL. (2008) Diabetic eNOS knockout mice develop distinct macro- and microvascular complications. *Lab Invest* 88(5):515–528.
5. Lee HH, Sanada S, An SM, Ye BJ, Lee JH, Seo YK, Lee C, Lee-Kwon W, Küper C, Neuhofer W, Choi SY, Kwon HM. (2016) LPS-induced NFκB enhanceosome requires TonEBP/NFAT5 without DNA binding. *Sci Rep* 6:24921.
6. Choi SY, Lee HH, Lee JH, Ye BJ, Yoo EJ, Kang HJ, Jung GW, An SM, Lee-Kwon W, Chiong M, Lavandero S, Kwon HM. (2016) TonEBP suppresses IL-10-mediated immunomodulation. *Sci Rep* 6:25726.
7. Yoon HJ, You S, Yoo SA, Kim NH, Kwon HM, Yoon CH, Cho CS, Hwang D, Kim WU. (2011) NFAT5 is a critical regulator of inflammatory arthritis. *Arthritis Rheum* 63(7):1843-1852.
8. Choi S, You S, Kim D, Choi SY, Kwon HM, Kim HS, Hwang D, Park YJ, Cho CS, Kim WU. (2017) Transcription factor NFAT5 promotes macrophage survival in rheumatoid arthritis. *J Clin Invest* 127(3):954-969.

9. Halterman JA, Kwon HM, Leitinger N, Wamhoff BR. (2012) NFAT5 expression in bone marrow-derived cells enhances atherosclerosis and drives macrophage migration. *Front Physiol* 3:313.
10. Yang B, Hodgkinson AD, Oates PJ, Kwon HM, Millward BA, Demaine AG. (2006) Elevated activity of transcription factor nuclear factor of activated T-cells 5 (NFAT5) and diabetic nephropathy. *Diabetes* 55(5):1450-1455.
11. Mitchell BD, McArdle PF, Shen H, Rampersaud E, Pollin TI, Bielak LF, Jaquish C, Douglas JA, Roy-Gagnon MH, Sack P, Naglieri R, Hines S, Horenstein RB, Chang YP, Post W, Ryan KA, Brereton NH, Pakyz RE, Sorkin J, Damcott CM, O'Connell JR, Mangano C, Corretti M, Vogel R, Herzog W, Weir MR, Peyser PA, Shuldiner AR. (2008) The genetic response to short-term interventions affecting cardiovascular function: Rationale and design of the Heredity and Phenotype Intervention (HAPI) Heart Study. *Am Heart J* 155(5):823–828.
12. Cheng YC, Kao WH, Mitchell BD, Sharrett AR, Ryan KA, Vogel RA, Shuldiner AR, Pollin TI. (2010) Genetic effects of postprandial variations of inflammatory markers in healthy individuals. *Obesity (Silver Spring)* 18(7):1417-1422.
13. Keyszer G, Lambiri I, Nagel R, Keysser C, Keysser M, Gromnica-Ihle E, Franz J, Burmester GR, Jung K. (1999) Circulating levels of matrix metalloproteinases MMP-3 and MMP-1, tissue inhibitor of metalloproteinases 1 (TIMP-1), and MMP-1/TIMP-1 complex in rheumatic disease. Correlation with clinical activity of rheumatoid arthritis versus other surrogate markers. *J Rheumatol* 26(2):251-258.
14. Barrett JC, Fry B, Maller J, Daly MJ. (2005) Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 21(2):263-265.
15. Köttgen A, Albrecht E, Teumer A, Vitart V, Krumsiek J, Hundertmark C, Pistis G, Ruggiero D, O'Seaghdha CM, Haller T, Yang Q, Tanaka T, Johnson AD, Kutalik Z, Smith AV, Shi J, Struchalin M, Middelberg RP, Brown MJ, Gaffo AL, Pirastu N, Li G, Hayward C, Zemunik T, Huffman J, Yengo L, Zhao JH, Demirkan A, Feitosa MF, Liu X,

Malerba G, Lopez LM, van der Harst P, Li X, Kleber ME, Hicks AA, Nolte IM, Johansson A, Murgia F, Wild SH, Bakker SJ, Peden JF, Dehghan A, Steri M, Tenesa A, Lagou V, Salo P, Mangino M, Rose LM, Lehtimäki T, Woodward OM, Okada Y, Tin A, Müller C, Oldmeadow C, Putku M, Czamara D, Kraft P, Frogheri L, Thun GA, Grotevendt A, Gislason GK, Harris TB, Launer LJ, McArdle P, Shuldiner AR, Boerwinkle E, Coresh J, Schmidt H, Schallert M, Martin NG, Montgomery GW, Kubo M, Nakamura Y, Tanaka T, Munroe PB, Samani NJ, Jacobs DR Jr, Liu K, D'Adamo P, Ulivi S, Rotter JI, Psaty BM, Vollenweider P, Waeber G, Campbell S, Devuyst O, Navarro P, Kolcic I, Hastie N, Balkau B, Froguel P, Esko T, Salumets A, Khaw KT, Langenberg C, Wareham NJ, Isaacs A, Kraja A, Zhang Q, Wild PS, Scott RJ, Holliday EG, Org E, Viigimaa M, Bandinelli S, Metter JE, Lupo A, Trabetti E, Sorice R, Döring A, Lattka E, Strauch K, Theis F, Waldenberger M, Wichmann HE, Davies G, Gow AJ, Bruinenberg M; LifeLines Cohort Study, Stolk RP, Kooner JS, Zhang W, Winkelmann BR, Boehm BO, Lucae S, Penninx BW, Smit JH, Curhan G, Mudgal P, Plenge RM, Portas L, Persico I, Kirin M, Wilson JF, Mateo Leach I, van Gilst WH, Goel A, Ongen H, Hofman A, Rivadeneira F, Uitterlinden AG, Imboden M, von Eckardstein A, Cucca F, Nagaraja R, Piras MG, Nauck M, Schurmann C, Budde K, Ernst F, Farrington SM, Theodoratou E, Prokopenko I, Stumvoll M, Jula A, Perola M, Salomaa V, Shin SY, Spector TD, Sala C, Ridker PM, Kähönen M, Viikari J, Hengstenberg C, Nelson CP; CARDIoGRAM Consortium; DIAGRAM Consortium; ICBP Consortium; MAGIC Consortium, Meschia JF, Nalls MA, Sharma P, Singleton AB, Kamatani N, Zeller T, Burnier M, Attia J, Laan M, Klopp N, Hillege HL, Kloiber S, Choi H, Pirastu M, Tore S, Probst-Hensch NM, Völzke H, Gudnason V, Parsa A, Schmidt R, Whitfield JB, Fornage M, Gasparini P, Siscovick DS, Polašek O, Campbell H, Rudan I, Bouatia-Naji N, Metspalu A, Loos RJ, van Duijn CM, Borecki IB, Ferrucci L, Gambaro G, Deary IJ, Wolffenbuttel BH, Chambers JC, März W, Pramstaller PP, Snieder H, Gyllensten U, Wright AF, Navis G, Watkins H, Witteman JC, Sanna S, Schipf S, Dunlop MG, Tönjes A, Ripatti S, Soranzo N, Toniolo D, Chasman DI,

Raitakari O, Kao WH, Ciullo M, Fox CS, Caulfield M, Bochud M, Gieger C. (2013) Genome-wide association analyses identify 18 new loci associated with serum urate concentrations. *Nat Genet* 45(2):145-154.