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Supplemental Information

Microbial Imidazole Propionate Affects Responses to Metformin through p38γ-Dependent Inhibitory AMPK Phosphorylation

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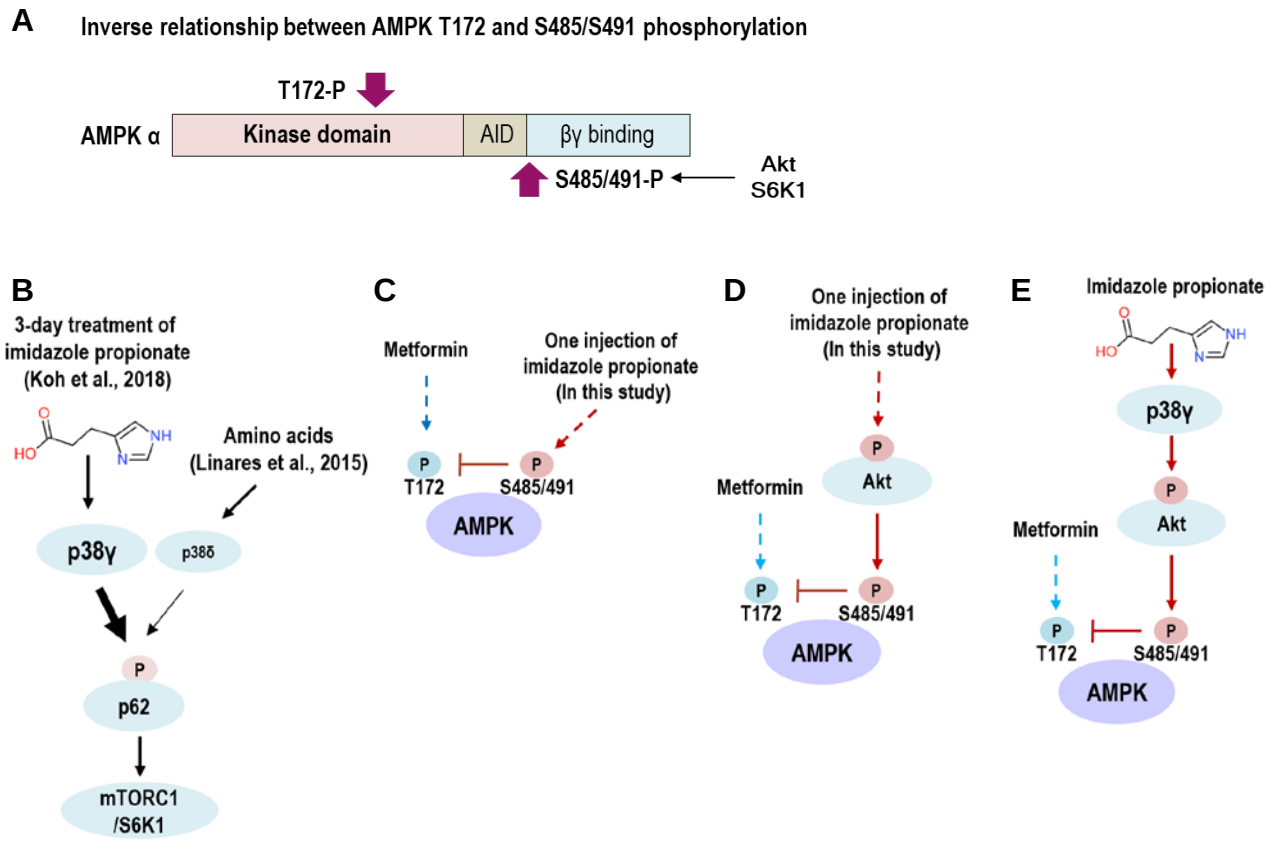


Figure S1. Schematic depiction of signalling pathways related to this study, Related to Figure 2 to 4

(A) Previously reported relationship between AMPK T172 and S485 phosphorylation.

(B) Previously reported signaling pathways activated after 3-day treatment with imidazole propionate or by amino acids.

(C-E) Signaling pathways investigated in this study.

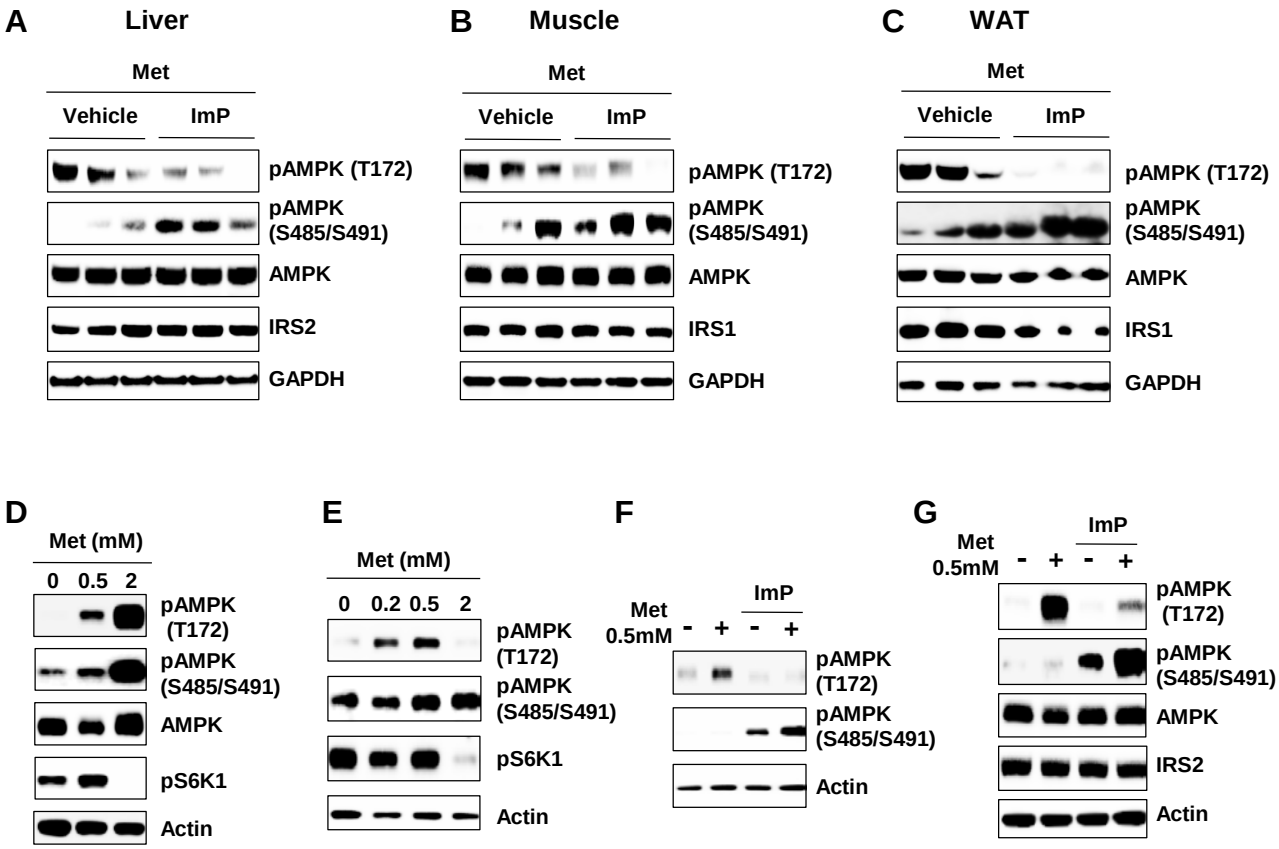


Figure S2. Inverse relationship between AMPK active site T172 and inhibitory S485 phosphorylation, Related to Figure 2

(**A-C**) Immunoblot of liver (**A**), soleus muscle (**B**), and WAT (**C**) lysates from mice showing effect of one intraperitoneal injection of imidazole propionate (ImP) on metformin (Met). Mice were injected intraperitoneally with vehicle (1% DMSO in water) or ImP (100 μg) and after 1 h metformin (200 mg/kg) was orally administered; tissues were collected 45 min after metformin administration (representative of n = 3).

(**D**) Concentration-dependent effects of Met on AMPK in primary hepatocytes. Serum-starved primary hepatocytes were incubated with the indicated concentrations of Met for 8 h (representative of n = 2).

(**E**) Concentration-dependent effects of Met on AMPK in HEK293 cells. Serum-starved HEK293 cells were incubated with the indicated concentrations of Met for 6 h (representative of n = 2).

(**F**) Inhibitory effect of ImP on Met-induced AMPK T172 phosphorylation. HEK293 cells were co-treated with ImP (100 μM) and Met for 6 h (representative of n = 3).

(**G**) Inhibitory effect of ImP on Met-induced AMPK T172 phosphorylation. Primary hepatocytes were co-treated with ImP (100 μM) and Met for 8 h (representative of n = 3).

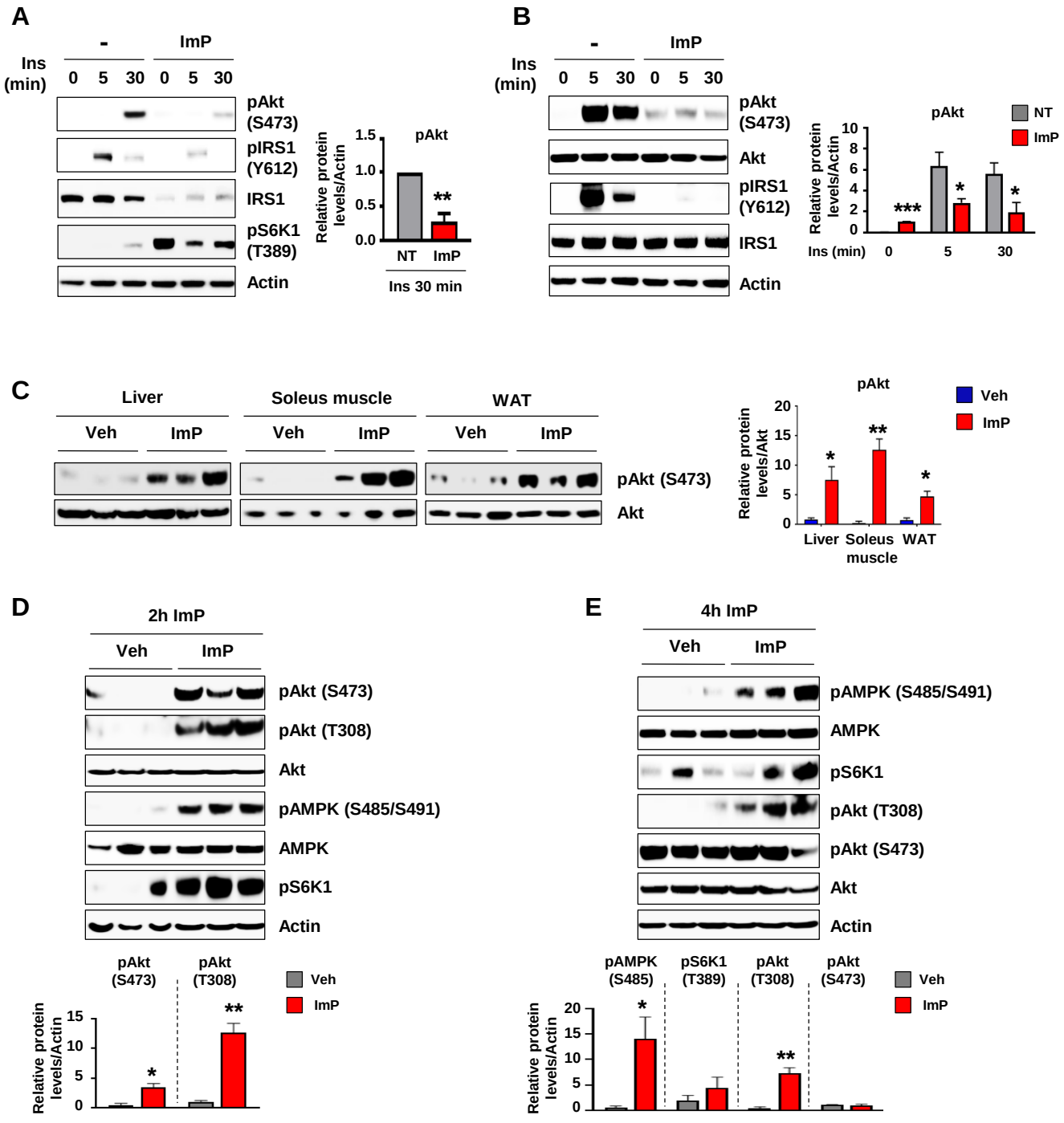

A
-
ImP
Ins (min)
0 5 30 0 5 30
pAkt (S473)
pIRS1 (Y612)
IRS1
pS6K1 (T389)
Actin
Relative protein levels/Actin
pAkt
1.5
1.0
0.5
0.0
**
NT
ImP
Ins 30 min
B
-
ImP
Ins (min)
0 5 30 0 5 30
pAkt (S473)
Akt
pIRS1 (Y612)
IRS1
Actin
Relative protein levels/Actin
pAkt
NT
ImP
10
8
6
4
2
0
***
*
*
Ins (min) 0 5 30
C
Liver
Soleus muscle
WAT
Veh
ImP
Veh
ImP
Veh
ImP
pAkt (S473)
Akt
pAkt
Veh
ImP
Relative protein levels/Akt
20
15
10
5
0
*
**
*
Liver
Soleus muscle
WAT
D
2h ImP
Veh
ImP
pAkt (S473)
pAkt (T308)
Akt
pAMPK (S485/S491)
AMPK
pS6K1
Actin
pAkt (S473)
pAkt (T308)
Veh
ImP
Relative protein levels/Actin
15
10
5
0
*
**
E
4h ImP
Veh
ImP
pAMPK (S485/S491)
AMPK
pS6K1
pAkt (T308)
pAkt (S473)
Akt
Actin
pAMPK (S485)
pS6K1 (T389)
pAkt (T308)
pAkt (S473)
Veh
ImP
Relative protein levels/Actin
20
15
10
5
0
*
**

Figure S3. Effects of imidazole propionate on basal Akt and inhibitory AMPK phosphorylation, Related to Figure 3

(A) Effect of imidazole propionate (ImP) or control (NT) on insulin-stimulated Akt phosphorylation when IRS reduction occurs. Serum-starved HEK293 cells were pretreated with 100 μM ImP for 8 h and then stimulated with 5 nM insulin (n = 3).

(**B)** Effect of 8 h ImP (100 μM) on Akt activation [basal and insulin (5 nM)-stimulated] before IRS reduction occurs in primary hepatocytes (n = 5).

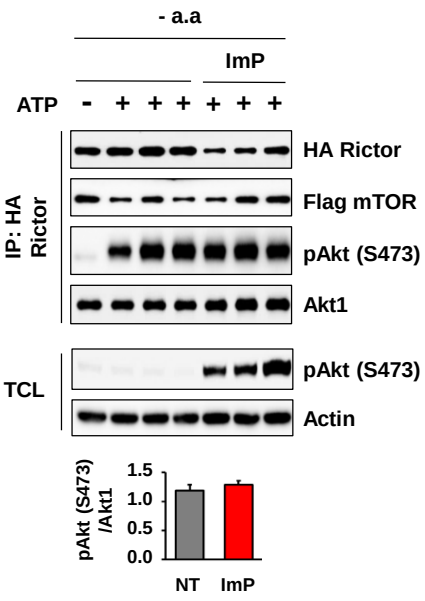
(**C**) Immunoblot of liver, soleus muscle, and WAT lysates from mice showing effect of 3-day treatment with vehicle (Veh) or 100 μg ImP (twice per day) on basal Akt activation (n = 3).

(**D)** Immunoblot of liver 2 h after one intraperitoneal injection of ImP (100 μg) in mice (n = 3).

(E) Immunoblot of liver 4 h after one intraperitoneal injection of ImP (100 μg) in mice (n = 3).

Data are mean ± s.e.m. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Unpaired two-tailed Student's *t*-tests (**A**-**E**).

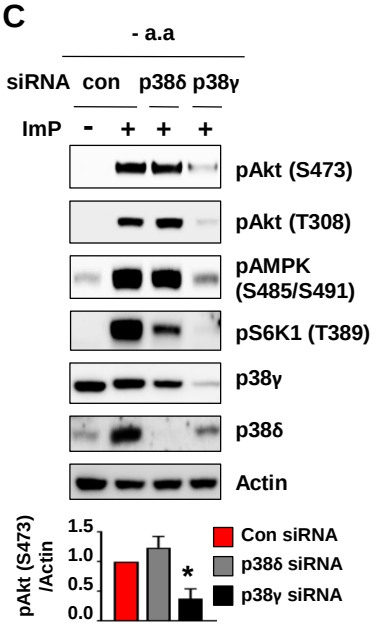
A



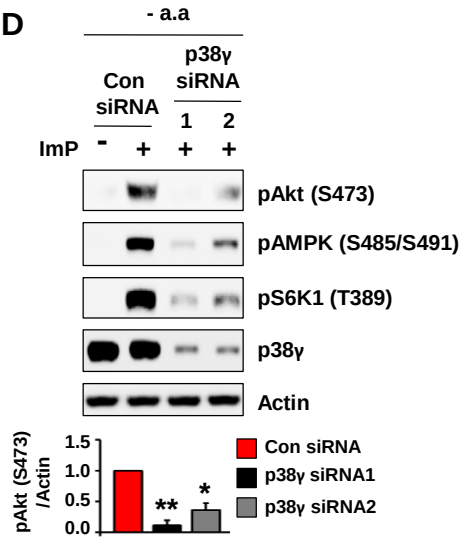
B

Akt1	Akt2	Akt3
mTOR	IKKε	p38δ
ERK5	CDK7	JNK2
JNK2	p38δ	ANPa
PITSLRE	TBK1	JNK1
CDK7	ANPa	JNK3
IKKε	PSKH1	CDK7
p38δ	ERK1	IKKε

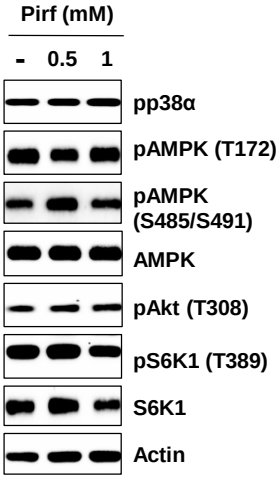
C



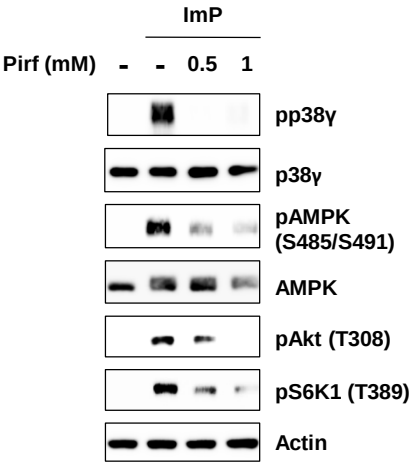
D



E



F



G

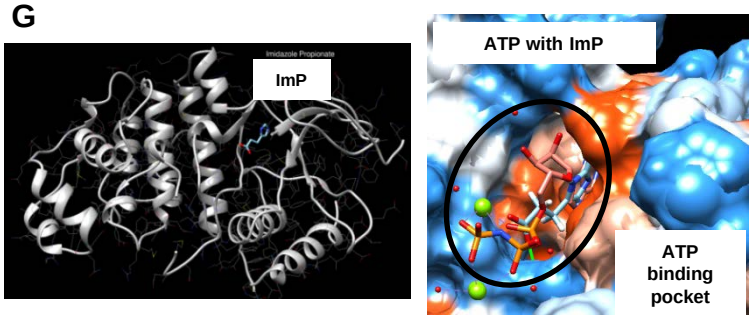


Figure S4. Role of p38γ on imidazole propionate-induced Akt/AMPK phosphorylation, Related to Figure 4

(A) Effects of imidazole propionate (ImP) on *in vitro* mTORC2 activity. HEK293 cells transfected with Flag mTOR and HA Rictor were serum- and amino acid-deprived and stimulated with ImP (100 μM) for 2 h; immunoprecipitated mTORC2 (via HA Rictor) was used as a kinase and inactive Akt1 was used as a substrate in an *in vitro* kinase assay (n = 6).

(B) Potential upstream kinase prediction towards S473 of Akt1, 2, and 3 according to Kinase Predictor V2 score-proximity from the PhosphoNET database.

(C) Effect of alternative p38 knockdown on ImP-induced Akt activation (n = 3). HEK293 cells were transfected with control siRNA (con siRNA), p38δ siRNA, or p38γ siRNA1 and incubated with 100 μM ImP in the absence of amino acids for 1.5 h.

(D) Effects of two distinct non-overlapping siRNAs against p38γ on ImP-induced Akt activation (n = 3). HEK293 cells were transfected with control siRNA (Con siRNA) or p38γ siRNA and incubated with 100 μM ImP in the absence of amino acids for 1.5 h.

(E) Effects of pirfenidone (Pirf) on AMPK in the absence of ImP. HEK293 cells were incubated with the indicated concentrations of Pirf for 6 h (representative of n = 2).

(F) Effects of the p38γ inhibitor pirfenidone (Pirf) on ImP-induced p38γ activation. Serum-starved HEK293 cells were co-incubated with100 μM ImP and Pirf for 6 h (representative of n = 2).

(G) Left, docking simulation of ImP (Zinc_24690773) to p38γ. **Right,** superimposition of p38γ-ATP on simulated p38γ-ImP. Molecular docking calculations were performed using SwissDock and binding modes were scored and ranked using FullFitness. Results were visualized by UCSF Chimera package.

Data are mean ± s.e.m. **P* < 0.05, ***P* < 0.01. Unpaired two-tailed Student's *t*-tests (**A, C, D**).

	Metformin successful (fasting glucose <7.8 mM)	**Metformin unsuccessful (fasting glucose ≥7.8 mM)**	*P* value
n	29	40	-
Men, n (%)	22 (75.9)	35 (87.5)	0.349
Age, years	67.8 ± 1.2	69.8 ± 0.9	0.251
Body mass index, kg/m^2	29.2 ± 0.6	30.8 ± 0.6	0.083
Fasting plasma glucose, mM	6.7 ± 0.2	9.9 ± 0.4	<0.001 (***)
Mean time from diagnosis, years	6.5 ± 1.6	7.4 ± 1.0	0.230

Table S1. Clinical characteristics of subjects with type 2 diabetes who were treated with metformin, Related to Figure 1

Data are presented as mean ± s.e.m or as proportions. *P* values were calculated using Wilcoxon Rank-Sum Test for continuous variables and chi square test for categorical variables.