

Metformin attenuates the onset of non-alcoholic fatty liver disease and affects intestinal microbiota and barrier in small intestine

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Supplementary Table S1. Effect of a chronic (6 weeks) and short-term feeding (4 days) of a FFC or control diet ± metformin on markers of inflammation and gluconeogenesis in liver tissue.

	groups				P-value		
	C	FFC	C+Met	FFC+Met	DExME	ME	DE
6 weeks							
<i>Il10</i> mRNA	100±5.6	114±7.7	79.5±8.1	158±12 ^{a,b,c}	<0.05	NS	<0.05
<i>Clec10a</i> mRNA	100±12	80.5±18	101±23	85.9±15	NS	NS	NS
<i>Pepck</i> mRNA	100±18	80.9±26	95.2±16	86.2±23	NS	NS	NS
4 days							
<i>Il10</i> mRNA	100±14	140±12	132±34	116±23	NS	NS	NS
<i>Clec10a</i> mRNA	100±18	92.0±6.5	138±32	108±15	NS	NS	NS
<i>Pepck</i> mRNA	100±31	50.4±10 ^c	118±20	31.3±4.8 ^c	NS	NS	<0.05

Values are means ± standard error of means and are shown as % of control; n=4-8. C: control diet, *Clec10a*: C-type lectin domain family 10 member A, C+Met: control diet and oral treatment with metformin (300 mg/kg BW/day), DE: diet effect, DExME: interaction between diet and metformin, FFC: fat-, fructose- and cholesterol-rich diet, FFC+Met: fat-, fructose- and cholesterol-rich diet and oral treatment with metformin (300 mg/kg BW/day). ME: metformin effect, Met: metformin, NS: not significant, *Il10*: interleukin 10, *Pepck*: phosphoenolpyruvate carboxykinase. ^a*P*<0.05 compared with mice fed a control diet; ^b*P*<0.05 compared with mice fed a FFC diet; ^c*P*<0.05 compared with mice fed a control diet treated with metformin.

Supplementary Table S2. Nutrient composition of control diet and fat-, fructose- and cholesterol-rich diet (FFC).

		Control	FFC
Crude protein (CP)	% wt/wt	17.4	16.0
Crude lipid (CL)	% wt/wt	5.1	11.8
Crude fibre	% wt/wt	5.0	2.0
Crude ash	% wt/wt	4.1	4.2
Corn Starch	% wt/wt	34.6	5.0
Sucrose	% wt/wt	11.0	-
Glucose	% wt/wt	-	5.0
Fructose	% wt/wt	-	50.0
Cholesterol	% wt/wt	-	0.16
L-Lysine	% wt/wt	1.43	1.32
L-Methionine + L-Cystein	% wt/wt	1.00	0.96
L-Threonine	% wt/wt	0.75	0.69
Calcium	% wt/wt	0.78	0.77
Phosphorus	% wt/wt	0.48	0.47
Sodium	% wt/wt	0.23	0.30
Vitamin A (retinol acetate)	IU/kg	15000	15000
Vitamin D₃ (cholecalciferol)	IU/kg	1500	1500
Vitamin E (α-tocopherol acetate)	mg/kg	150	150
Fatty acids			
C4:0	% wt/wt	-	0.44
C6:0	% wt/wt	-	0.29
C8:0	% wt/wt	-	0.16
C10:0	% wt/wt	-	0.35
C12:0	% wt/wt	-	0.40
C14:0	% wt/wt	0.02	1.23
C16:0	% wt/wt	0.57	3.10
C18:0	% wt/wt	0.18	1.14
C18:1 (n-9)	% wt/wt	1.28	2.58
C18:2 (n-6)	% wt/wt	2.65	0.21
C18:3 (n-3)	% wt/wt	0.29	0.06
Metabolizable energy	kcal/kg	3752	4254
CP	kcal%	19	15
CL	kcal%	12	25
Carbohydrate	kcal%	69	60

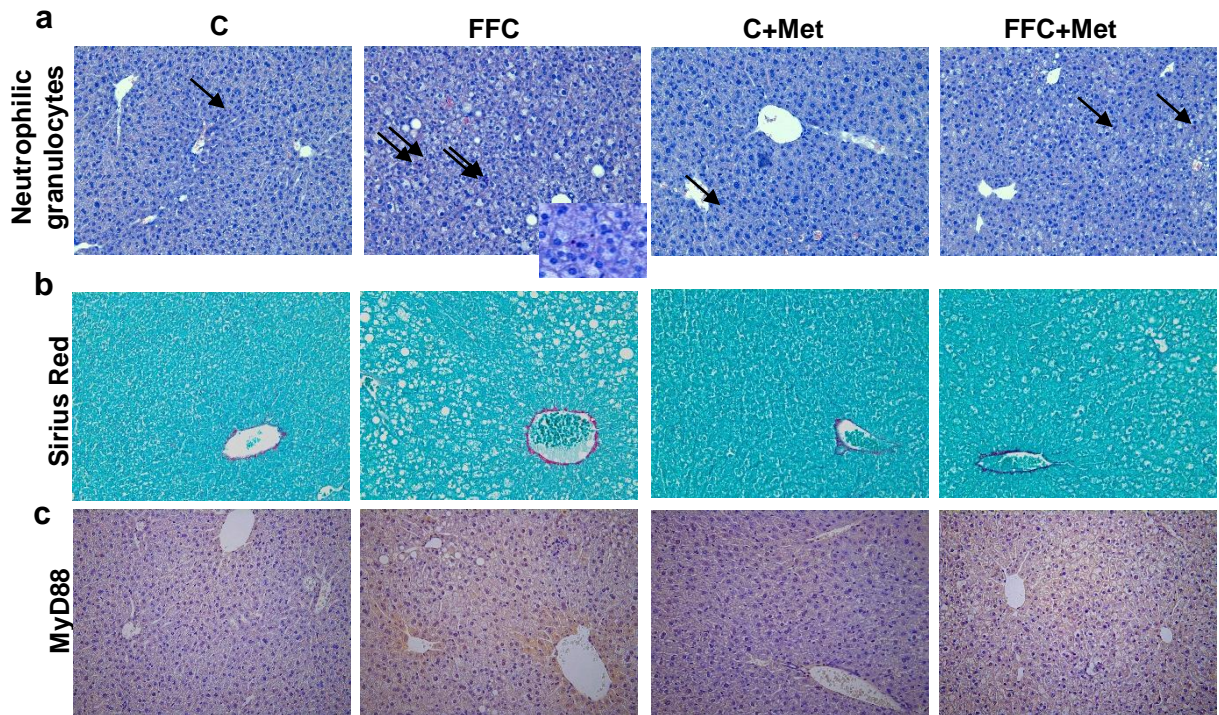
CL: crude lipid, CP: crude protein

Supplementary Table S3. Primer sequences.

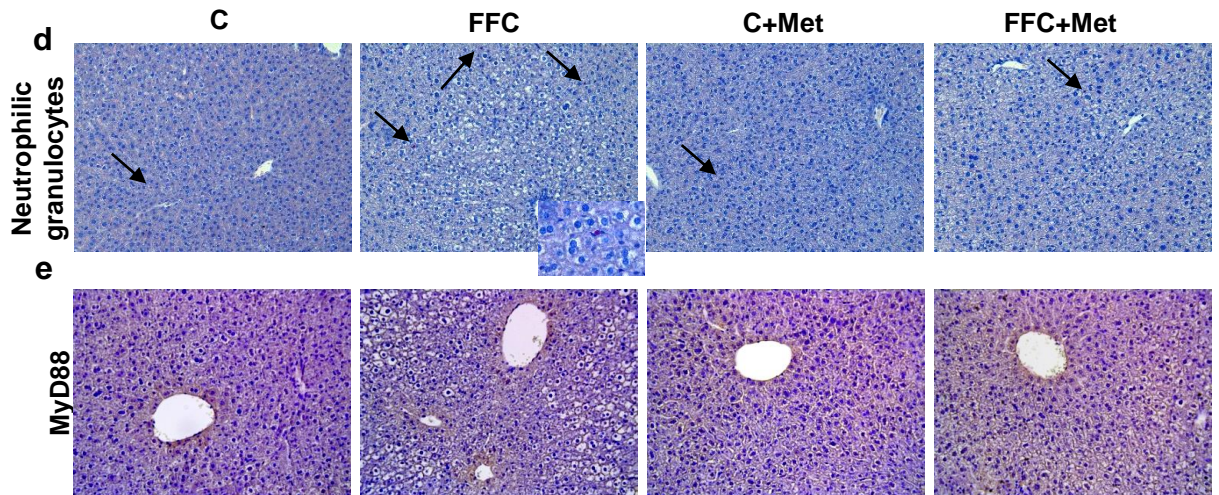
	Forward (5'-3')	Reverse (5'-3')
18S	GTA ACC CGT TGA ACC CCA TT	CCA TCC AAT CGG TAG TAG CG
Acc	CTT CCT CCT GAT CAG CAA CTC T	CGT GAG TTT TCC CAA AAT AAG C
Acox1	GGG AAT TTG GCA TCG CAG AC	CAT CTC CGT CTG GGC GTA GG
asma	CTG ACA GAG GCA CCA CTG AA	CAT CTC CAG AGT CCA GCA CA
Clec10a	TGG TCG TCT CCG TGA TTG GA	CCT GCA GTT CCT GCC TGT GA
Eef2	GTG ACA GCT GCC TTG CGT GT	GAT GCG CTG GAA GGT CTG GT
Fasn	TCT GGG CCA ACC TCA TTG GT	GAA GCT GGG GGT CCA TTG TG
Il10	CAG GGC CCT TTG CTA TGG TG	CGG CTG GGG GAT GAC AGT AG
Il1b	TGG CTG TGG AGA AGC TGT GG	GTC CGA CAG CAC GAG GCT TT
Mmp13	AGA AGT GTG ACC CAG CCC TA	GCG CAA GAA GAA TCT GTC TTT
Occ	CAT CAG CCA TGT CCG TGA GG	GGG GCG ACG TCC ATT TGT AG
Pepck	CCC TGG GAG ATG GGG AGT TC	CCC ACC ATA TCC GCT TCC AA
Sreb1c	ACC GGC TAC TGC TGG ACT GC	AGA GCA AGA GGG TGC CAT CG
Tlr4	AGC CAT TGC TGC CAA CAT CA	GCT GCC TCA GCA GGG ACT TC
Vimentin	GCC GCA GCC TCT ATT CCT CA	CTC GAG GAA GAG CAC CTT GT
Zo1	GCA GAC TTC TGG AGG TTT CG	CTT GCC AAC TTT TCT CTG GC

Acc: acetyl coA carboxylase, Acox1: acyl coA oxidase-1, asma: α -smooth muscle actin, Clec10a: C-type lectin domain family 10 member A, Eef2: eukaryotic elongation factor 2, Fasn: fatty acid synthase, Il10: interleukin 10, Il1b: interleukin 1 β , Mmp: matrix-metalloproteinase, Occ: occludin, Pepck: phosphoenolpyruvate carboxykinase, Sreb1c: sterol regulatory element-binding protein 1, Tlr: toll-like receptor, Zo1: zonula occludens-1

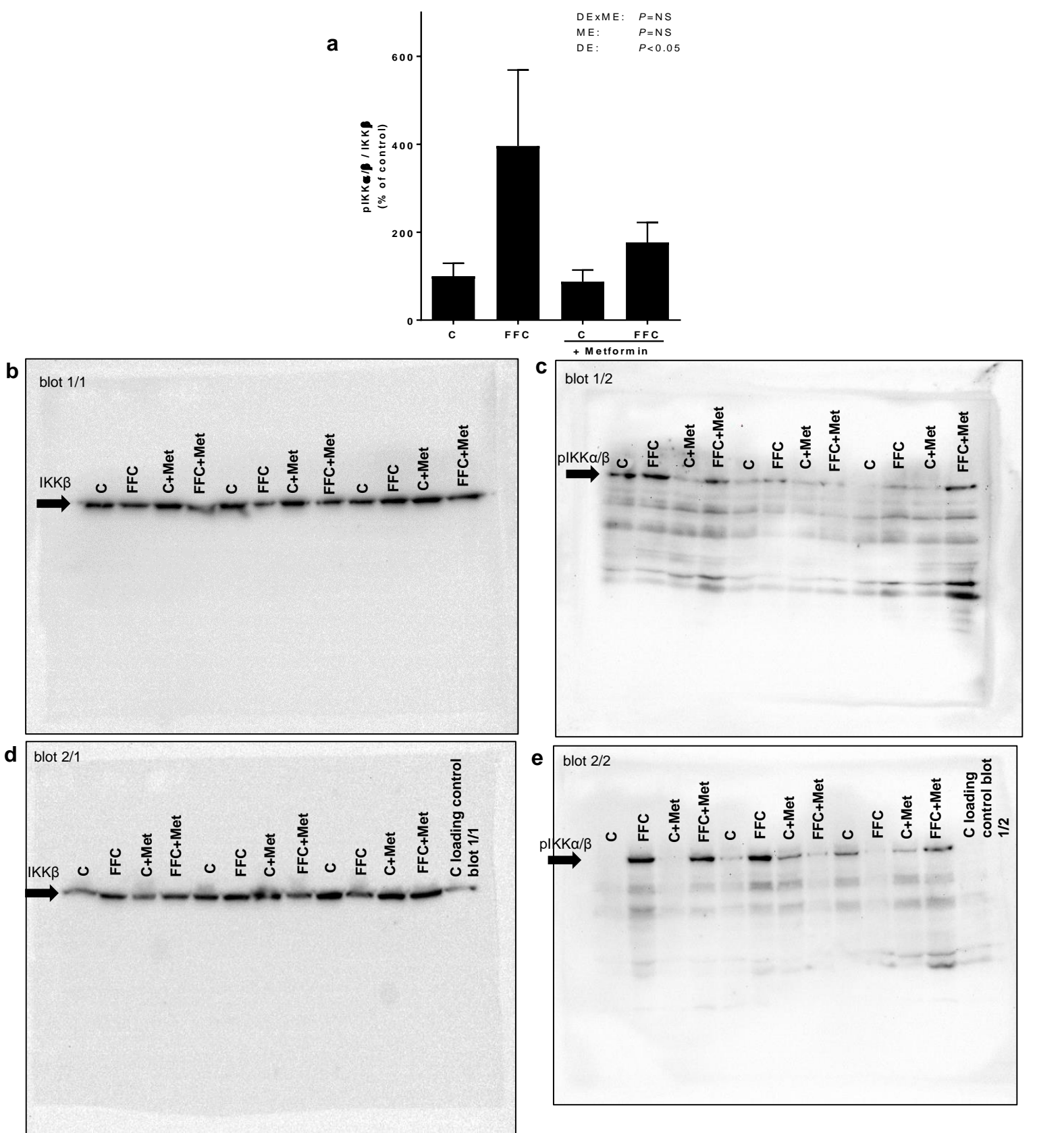
6 weeks



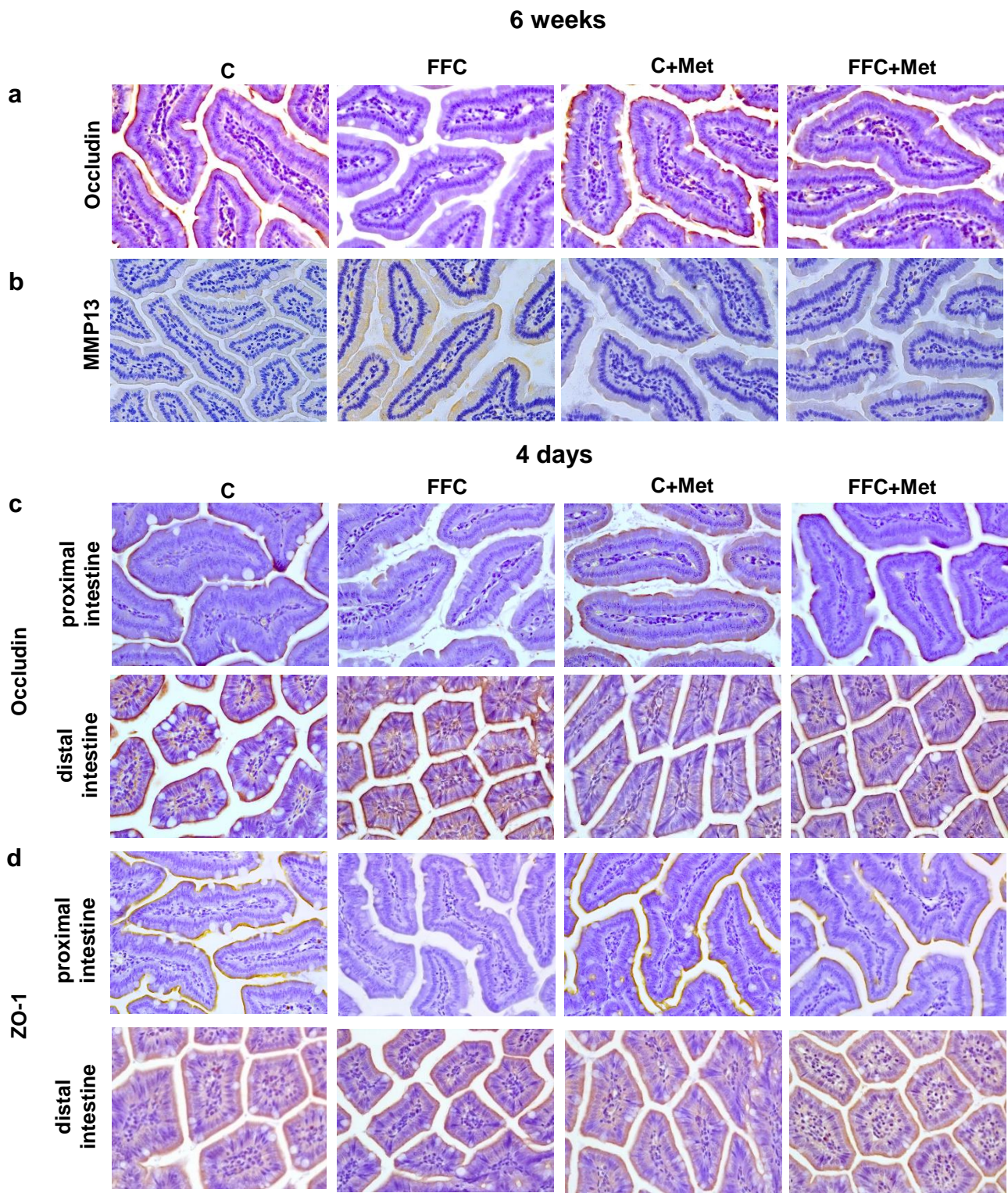
4 days



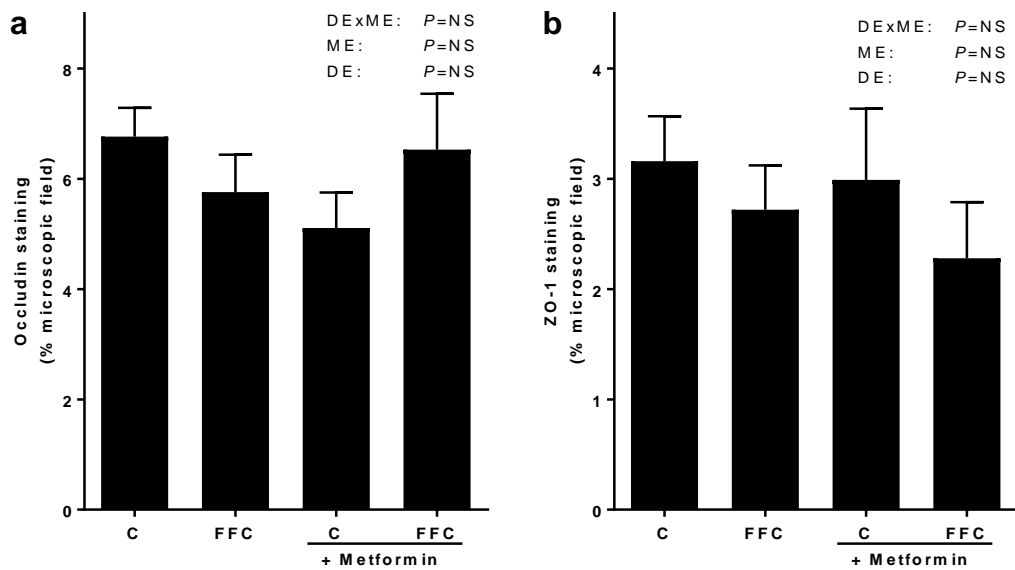
Supplementary Figure S1: Representative pictures of neutrophilic granulocytes, Sirius Red staining and MyD88 protein staining in long-term feeding (6 weeks) and short-term feeding (4 days) of a FFC or control diet \pm metformin. (a,d) Neutrophilic granulocytes in liver sections (200x; arrows are indicators for neutrophilic granulocytes; insertion in FFC sections 630x), (b) Sirius red staining in liver section and (c, e) MyD88 in liver tissue (200x) in long-term feeding and short-term feeding. C: control diet; C+Met: control diet and oral treatment with metformin (300 mg/kg BW/day); FFC: fat-, fructose- and cholesterol-rich diet; FFC+Met: fat-, fructose- and cholesterol-rich diet and oral treatment with metformin (300 mg/kg BW/day); MyD88: myeloid differentiation primary response 88.



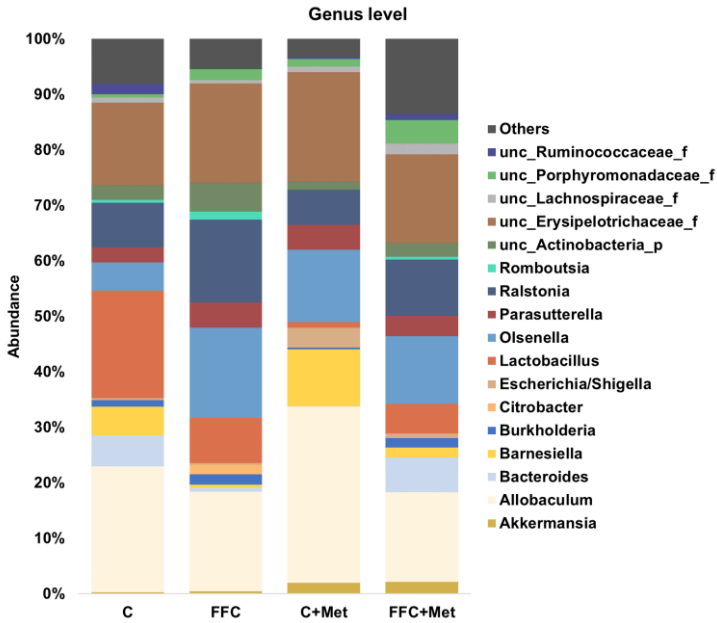
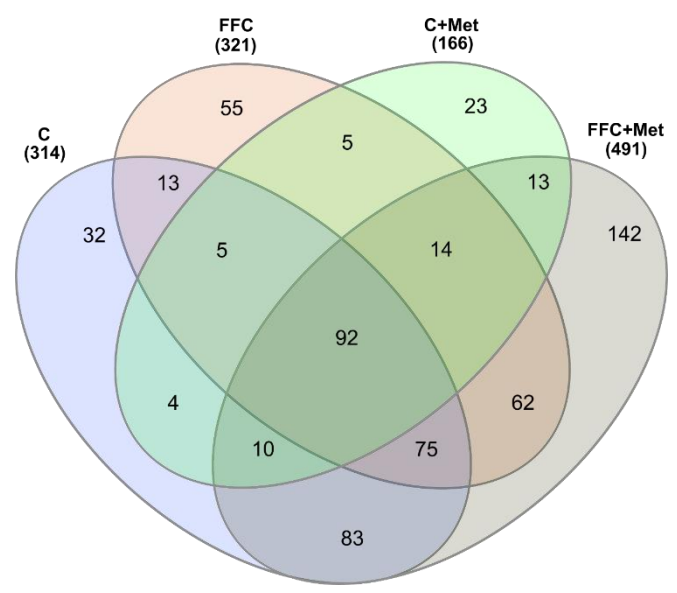
Supplementary Figure S2: Effect of a long-term feeding (6 weeks) of a FFC or control diet \pm metformin on phosphorylation status of inhibitor of nuclear factor kappa-B kinase subunit α and β (pIKK α/β) in liver tissue. (a) Densitometric analysis of pIKK α/β and IKK β Western blot and full length blot of (b,d) IKK β and (c,e) pIKK α/β ; $n=5-6$; samples derive from the same experiment and blots were processed in parallel. Data presented as means \pm standard error of means. C: control diet; C+Met: control diet and oral treatment with metformin (300 mg/kg BW/day); DE: diet effect; DExME: interaction between diet and metformin; FFC: fat-, fructose- and cholesterol-rich diet; FFC+Met: fat-, fructose- and cholesterol-rich diet and oral treatment with metformin (300 mg/kg BW/day); ME: metformin effect ; NS: not significant.



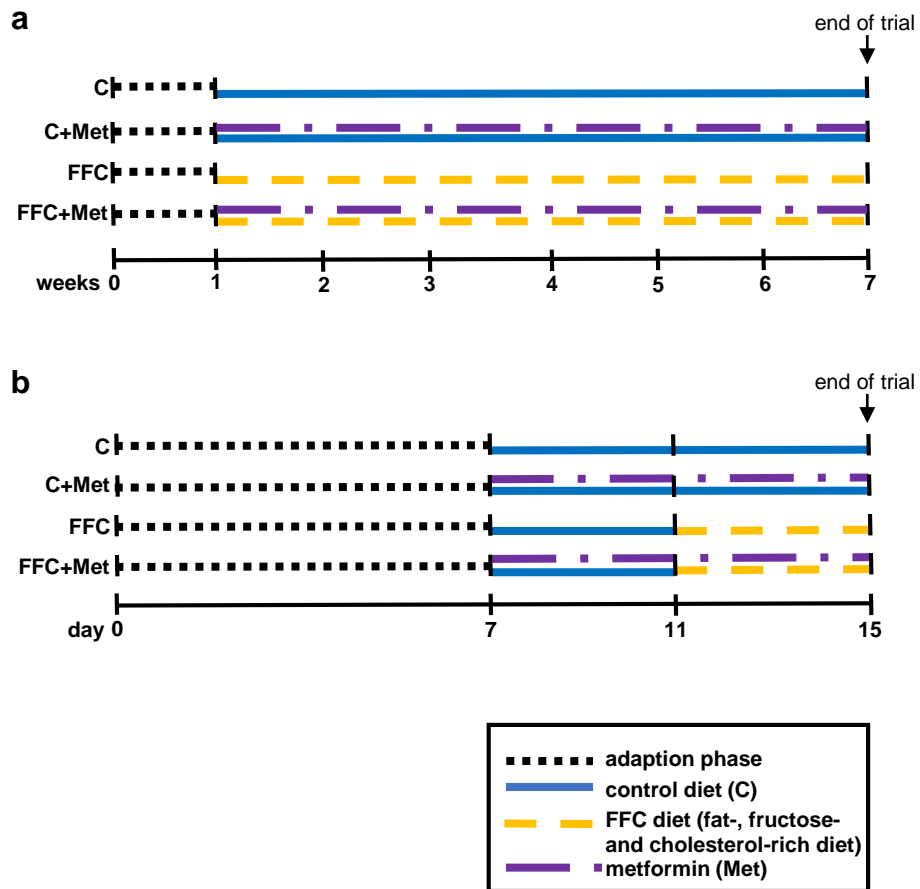
Supplementary Figure S3: Representative pictures of immunohistochemical stainings in a long-term feeding (6 weeks) and short-term feeding (4 days) of a FFC or control diet ± metformin. (a) Occludin (400x) and (b) MMP13 (400x) protein staining in proximal intestinal tissue in long-term trial as well as (c) occludin (400x) and (d) ZO-1 (400x) in proximal and distal intestinal tissue in short-term trial. C: control diet; C+Met: control diet and oral treatment with metformin (300 mg/kg BW/day); FFC: fat-, fructose- and cholesterol-rich diet; FFC+Met: fat-, fructose- and cholesterol-rich diet and oral treatment with metformin (300 mg/kg BW/day); MMP13: matrix-metalloproteinase 13; ZO-1: zonula occludens-1.



Supplementary Figure S4: Effect of a short-term feeding (4 days) of a FFC or control diet \pm metformin on tight junction proteins in distal small intestine. Densitometric analysis of (a) occludin and (b) ZO-1 protein staining in tissue of distal small intestine; $n=7-8$. Data presented as means \pm standard error of means. C: control diet; C+Met: control diet and oral treatment with metformin (300 mg/kg BW/day); DE: diet effect; DExME: interaction between diet and metformin; FFC: fat-, fructose- and cholesterol-rich diet; FFC+Met: fat-, fructose- and cholesterol-rich diet and oral treatment with metformin (300 mg/kg BW/day); ME: metformin effect ; NS: not significant; ZO-1: zonula occludens-1.

a**b**

Supplementary Figure S5: Effect of a short-term feeding (4 days) of a FFC or control diet ± metformin on microbial community in small intestine. (a) At genus level. (b) Venn diagram with OTUs present in the samples of each diet; n=4-6. C: control diet; C+Met: control diet and oral treatment with metformin (300 mg/kg BW/day); FFC: fat-, fructose- and cholesterol-rich diet; FFC+Met: fat-, fructose- and cholesterol-rich diet and oral treatment with metformin (300 mg/kg BW/day); unc: unclassified.



Supplementary Figure S6: Summary of study design. (a) Chronic feeding trial over 6 weeks and (b) short-term feeding trial fed the FFC or control diet over 4 days \pm metformin. C: control diet; C+Met: control diet and oral treatment with metformin (300 mg/kg BW/day); FFC: fat-, fructose- and cholesterol-rich diet; FFC+Metformin: fat-, fructose- and cholesterol-rich diet and oral treatment with metformin (300 mg/kg BW/day).