Supplementary Material (SM) to:

Susceptibility loci for adiposity phenotypes on 8p, 9p and 16q in American Samoa and Samoa

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Subjects and Methods

Study Sample

This study includes 71 pedigrees containing 3,016 individuals, age ≥18 years, (1,523 males and 1,493 females). The number of members in each pedigree span from 3 to 719 individuals (42.48 in average), and the number of generations in each pedigree span from 2 to 8 (3.87 in average). Each family has at least two genotyped individuals. The largest family has 246 genotyped individuals. All together 1,164 individuals (534 males and 630 females) were genotyped. All participants included in our previous genome scans of American Samoa (34 families) (1) and Samoa (46 families) (2) are also included in this study. In addition, one small family, that was large enough for investigation only if individuals from both polities were studied, was included in this study. Twenty of the families include genotyped family members from American Samoa as well as from Samoa. In average the pedigrees collected from American Samoa (45.94 individuals/family, ranging from 3 to 604) (1) tend to be larger than the pedigrees from the Samoa (35.09 individuals/family, ranging from 3 to 222) (2).

The participants in this study as well as in our previous two genome-wide scans (1, 2) of American Samoa and Samoa, respectively, took part in the Samoan Family Study of Overweight and Diabetes in 2002-03 (3). Recruitment in American Samoa, in 2002, was based on random selection of probands seen in the 1990-94 cohort study (4, 5), and the presence of at least two adult siblings alive and residing in American Samoa. Recruitment in Samoa, in 2003, was first based on finding individuals in Samoa who were members of American Samoa pedigrees who had been recruited in 2002. We then selected villages throughout the nation to assess geographic and economic diversity, and

chose families based on maximum number of available adult siblings. Participants in the 1990-94 cohort study (which was originally designed as a longitudinal study of blood pressure change over time) had to: 1) not have a medical diagnosis of hypertension or type 2 diabetes (based on doctor's report or current use of medications for either); and 2) self-report that all four grandparents originated from American Samoa. Probands and families were unselected for obesity or related phenotypes. Protocols for this study were approved by: the Brown University Institutional Review Board; American Samoan Institutional Review Board; and the Government of Samoa, Ministry of Health, Health Research Committee. Written informed consent was obtained from all participants.

Phenotypes

Standard anthropometric techniques and measurements were used to measure stature, weight and abdominal circumferences (ABDCIR), and to calculate body mass index (BMI). Bioelectrical impedance measures of resistance were obtained with the RJL Systems, Inc. BIA-101Q device (Clinton, MI 48035), using standard procedures. These measures were then used to calculate body fat percentage (%BF) using equations established from body composition studies in Samoans (3, 6). During field work an unexpected breakdown of the RJL System caused non-random missingness of %BF from the data collection in Samoa. Fasting blood specimens were drawn after a 10-hour minimum overnight fast into Vacutainers, separated with a portable field centrifuge and stored in plastic storage tubes at -40^{o} in local freezers. Serum was shipped on dry ice to Providence, RI, where serum leptin (LEPTIN) was assayed by radioimmunoassay (RIA) using a kit from ALPCO (Windham, NH) and serum adiponectin (ADIPONEC) was assayed using RIA kits from Linco, Inc. (St. Charles, MI).

Genotypes

Genomic DNA was isolated from 10 ml of EDTA blood sample using the Puregene Kit (Gentra Systems Inc., Minneapolis, MN). DNA was quantified, diluted to 20 μg/ml and arrayed in 96 well microtitre plates for storage. Microsatellite markers in the ABI PRISM linkage mapping set v2.5 MD10 (Applied Biosystems Inc., Foster City, CA) with an average spacing of approximately 10 cM were genotyped. Multiplex PCRs, of three to five markers, were performed in a 96 well format on a GeneAmp® PCR machine (Applied Biosystems Inc., Foster City, CA). Each 96-well PCR contained two wells with positive controls (CEPH DNA 1347-02) and one well with negative control (distilled water). Amplicons from four 96-well plates were assembled in to 384-well format using Biomek FX liquid handling station (Beckman Coulter, Fullerton, CA) prior to separation. Amplicons from microsatellite panel 8, 9, 10, 12 and 28 from the American Samoan samples were separated on an ABI PRISM 3100 genetic Analyzer (Applied Biosystems Inc., Foster City, CA) using internal size standard ROX400^{TM} (Applied Biosystems Inc., Foster City, CA) and amplicons from all other panels from the American Samoan samples as well as from the Samoan samples were separated on an ABI PRISM 3130XL genetic Analyzer (Applied Biosystems Inc., Foster City, CA) using internal size standard 500LIZ ^{TM} (Applied Biosystems Inc., Foster City, CA). To minimize miscalled genotypes, allele labels were assigned using the GeneMapper V4.0 software and checked manually by two persons.

Error Checking and Data Handling

The sample sets, from American Samoa and Samoa, have been extensively checked for genotype errors and errors of pedigree structure prior to the separate genome

scans for the two data sets (1, 2). To detect genotype errors, 'reasonable' allele size, number of alleles, allele frequencies and divergence from expected heterozygosity rates were checked for. The PEDSYS database system (PEDSYS, Southwest Foundation for Biomedical Research) was used to prepare the pedigree structure file based on self-reported pedigree information. To detect errors in pedigree structure, PEDSTATS (7) was used to check for internal consistency of ages, and RELPAIR v2.0.1 (8, 9) and PREST (10, 11) were used to check the accuracy of the self-reported pedigree relationships.

For the combined sample set, including American Samoans and Samoans, used in this study we performed additional checks for inconsistencies in pedigree structure using RELPAIR v2.0.1 (8, 9).

The "set correct_errors 1" option in LOKI (12) was used to remove a minimal set of genotypes to generate Mendelianly consistent pedigrees for the autosomes. For the X chromosome we used the option in Mega2 (13) to exclude Mendelian errors. Mega2 and the statistical software R (The R Project for Statistical Computing) were used interactively to set up files for the analyses performed in this study.

Multipoint Linkage Analysis

Univariate multipoint linkage analysis

Since the multipoint variance linkage analysis is sensitive to deviations of trait distributions from multivariate normality we, in addition to Box-Cox power transformation (14), used the multivariate t-distribution in SOLAR (15, 16) to guard against false positives due to possible non-normality for each phenotype.

In this study, three sets of covariates were used for multipoint linkage analyses (Table 1, in main text). In the basic set, only age, sex and originating polity were

included. Each covariate was screened for statistical significance using SOLAR. Only covariates with relatively strong effects ($p \leq 0.10$) were included in the final model. In an attempt to increase the power of our study by adjusting for specific environmental factors influencing the traits, we used the environmental set, including farm work, smoking and years of education in addition to age, sex and originating polity. When adjusting for the significant covariates in the basic set and in the environmental set, the kurtosis residual for the five investigated phenotypes, ABDCIR, ADIPONEC, BMI, %BF and LEPTIN, were all within normal range (<0.8). However, when adjusting LEPTIN, ADIPONEC and ABDCIR for the extended set, including BMI in addition to the covariates in the environmental set, we detected kurtosis residuals out of range (1.6-3.3) for the three investigated traits. In our previous studies of American Samoans and Samoans, we used a covariate set including %BF instead of BMI in addition to the three sets described above. Here we have not adjusted for %BF since including %BF as a covariate would cause a non-random missingness with respect to polity due to the low number of individuals phenotyped for %BF in Samoa.

A LOD score ≥3.3 was taken as evidence of significant linkage, which is equivalent to a p-value of 0.0001 or less. A LOD score ≥1.9 and LOD score ≥1.175 were considered to show evidence of suggestive linkage and potential linkage, respectively (17).

Bivariate analysis

For the bivariate linkage analysis, as well as for the univariate linkage analysis, a likelihood-ratio test for linkage is carried out with SOLAR. In contrast to the univariate LOD score, the bivariate LOD score asymptotically has two degrees of freedom. Thus,

the bivariate LOD score is not directly comparable to traditional univariate LOD score. As described previously (1, 2) the bivariate LOD score was converted to 1 degree of freedom before it was compared to traditional LOD scores. We assumed that Rhoq (QTL-specific genetic correlation between the two traits) is constrained when converting the bivariate LOD score (18, 19) and used the "loddf –default –c_rhoq" command in SOLAR for this conversion.

Results

Recalled Alleles

After initial alignment using our merging strategy, genotypes at ten markers from the two study samples did not align satisfactory. Nine of these markers were not affected by the use of different genotyping instruments and could therefore be recalled using bin patterns created from genotypes from the two study samples simultaneously, instead of bin patterns created from each data set respectively, which initially were used. The use of a new bin pattern created from all genotyped individuals slightly moved the location of the bin borders for some parts of the bin pattern which was enough for satisfactory alignment of the two study samples. For the 10th marker in need of recalling, which was genotyped using different instruments in the two study samples, the recalling was done using uniquely designed bin patterns for the specific instruments and the specific study samples. In total, 373 autosomal microsatellite markers and 14 X chromosomal markers were genotyped in the samples from the two polities.

Interaction between Covariates

As mentioned in the main text, when we were about to finalize this manuscript comments from an independent scientist motivated us to investigate the effect allowing for interaction between covariates on the results by performing univariate multipoint linkage analysis, as previously described, using two additional covariate sets, the "basic set with interaction" and the "environmental set with interaction". The "basic set with interaction" included the five covariates age, sex, age^2, age*sex and age^2*sex as well as polity of residence and interaction between polity and the five covariates, one at a time. When investigating the effect of interaction between the environmental covariates including farm work, education and smoking with the covariates in the "basic set with interaction" we were not able to perform screening for significance to all possible covariate combinations without running into problems using SOLAR. Therefore the "environmental set with interaction" was limited to include farm work, education and smoking as well as interaction between any of these three covariates, one at a time, with age, sex or polity of residence in addition to the eleven covariates included in the "basic covariate set with interaction". Heritability estimates and significant covariates included in polygenic model for each trait are shown in SM, Table S4. SM, Table S5 shows multipoint LOD scores ≥ 1.9 for the two covariate sets with interaction. Even though the LOD scores are slightly altered, the overall interpretation of the results, with strongly suggestive linkage on chromosome 9p and 16q to adiposity-related phenotypes, still remains essentially the same.

Authors contributions

KÅ, under the supervision of DEW, developed the strategy of merging genotypes, carried out the statistical analyses of the data and wrote most of the manuscript. FD, under the supervision of DEW, provided valuable input on statistical analyses. GS performed corrections of questionable allele calls detected when genotypes were merged. EDK, under the supervision of STM, performed all phenotype data management, checking and correction of the reported pedigrees, and participated in both years of fieldwork. GS, SRI, QZ and DS, under the supervision of RD, performed DNA extraction, genotyping and genotype data cleaning. STR, under the supervision of STM, was field director in both locations, and performed initial data management of phenotypic and pedigree data. JT and SV provided guidance in conduct of the fieldwork in American Samoa and Samoa, respectively. RD and LJ participated in designing the molecular work, quality control, data-management and supervised the analysis of the raw genotyping data. STM, RD, and DEW collaboratively designed and led this study and obtained funding for it. All the authors read and approved the final manuscript.

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Figure Legends:

Figure S1. Alignments of markers with S values >0.10 after shift, if needed, are shown to the left. Corresponding unshifted marker alignments are shown to the right. American Samoan frequencies are shown in black and Samoan in grey.

Figure S2. Multipoint univariate linkage results. The left column shows linkage results adjusted for the basic set including age, sex and polity of residence. The middle column shows linkage results screened for the environmental set including age, sex, polity of residence, farm work, education and smoking. The right column shows linkage results screened for the extended set including age, sex, polity of residence, farm work, education and smoking as well as for BMI. Significance level for suggestive linkage is indicated with horizontal line (LOD 1.9).

Figure S3. Plots of genome-wide multipoint LOD score detected in the American Samoan sample, the Samoan sample and in the combined study samples. **a)** Screened for the basic covariate set. **b)** Screened for the environmental covariate set. The level of genome-wide significance (LOD ≥3.3) is indicated with dotted lines, and the level for suggestive (LOD ≥1.9) and potential LOD scores (LOD ≥1.17) are indicated with dashed and solid lines, respectively.

Table S1: Characteristics of non-transformed phenotypes measured in study participants

Phenotype	N[a]	Am. Samoa Mean (±s.d.)		Samoa Mean (±s.d.)		Combined Mean (±s.d.)	
		Males	Females	Males	Females	Males	Females
Age (years)	1269	43.2±16.5	43.0±16.1	41.8±16.3	45.2±17.4	42.4±16.4	44.1±16.8
Sex (%)	1269	44	56	50	50	47	53
Education (years)	1234	11.7±2.4	12.0±2.4	9.7±3.4	10.1±3.0	10.6±3.1	11.0±2.9
Farmer (yes/no %)	1267	53/47	23/77	84/16	30/70	70/30	26/74
Smoker (yes/no %)	1128	38/59	20/74	38/50	13/67	38/53	17/71
Body mass index (BMI) (kg/m^2)	1263	33.5±7.6	36.6±8.4	28.9±5.4	33.0±7.6	30.9±6.9	34.8±8.2
Percent Body fat (%BF)	1007	33.7±6.7	41.6±6.3	28.4±7.2	39.2±6.8	31.3±7.4	40.7±6.6
Abdominal circumference (ABDCIR) (cm)	1267	107.8±16.3	111.3±16.5	95.9±15.1	106.9±16.3	101.1±16.7	109.1±16.6
Leptin (LEPTIN) (ng/ml)	1226	11.4±9.9	30.3±16.0	6.3±6.6	24.1±13.7	8.6±8.6	27.2±15.2
Adiponectin (ADIPONEC) (µg/ml)	1219	8.3±6.3	11.1±10.0	9.9±7.8	12.9±8.3	9.2±7.2	12.0±9.2

[a] Total number of phenotyped individuals.

Table S2: Heritability estimates (h^2) of investigated traits and variance explained by covariates

Trait	h^2 (s.e.) (screened for Basic Set)[a]	Variance[b] (%)	h^2 (s.e.) (screened for Environmental Set)[a]	Variance[b] (%)	h^2 (s.e.) (screened for Extended Set)[a]	Variance (%)
ABDCIR	0.42 (0.06) – A,S,R	19.7	0.39 (0.06) - A,S,R,F,E	20.1	0.33 (0.06) - A,F,B	87.8
ADIPONEC	0.45 (0.07) – A,S,R	13.2	0.47 (0.07) - A,S,R,E,C	11.0	0.52 (0.07) - A,S,R,C,B	21.0
LEPTIN	0.44 (0.06) – A,S,R	50.5	0.34 (0.07) - A,S,R,F,E,C	53.6	0.31 (0.08) - S,R,F,C,B	82.8
BMI	0.45 (0.06) – A,S,R	13.3	0.41 (0.06) - A,S,R,F,E	13.5	_	_
%BF	0.45 (0.07) – A,S,R	39.3	0.42 (0.07) - A,S,R,F,E	40.9	_	_

h^2 for all traits are significantly different from zero (p-value $<10^{-5}$). [a] Significant covariates (p-value <0.1) included in polygenic model are presented: A=age, S=sex, R=polity of residence, F=farm work, E=education, C=cigarette smoker and B=body mass index. [b] Variance explained by included covariates.

Table S3: Summary of chromosomal regions with suggestive linkage (LOD ≥1.9) in at least two of the three studied sample sets from the Samoan islands

Cytogenetic position	Trait	Am. Samoa[a]		Samoa[a]		Combined[a]	
9p22-p21	%BF			1.76	A,S	**2.48**	A,S,R,F,E
	ABDCIR			**2.14**	A,S,E	**2.14**	A,S,R,F,E
13q31.3-q33.1	BMI			**2.09**	A,S,E		
	%BF			1.62	A,S		
	ABDCIR			1.66	A,S,E		
	LEPTIN			**2.30**	A,S,E,C	1.67	A,S,R,F,E,C
	ADIPONEC	**2.41**	A,S,F,E				
16q21-q23	BMI	1.57	S				
	%BF	**2.24**	A,S				
	ABDCIR	**1.95**	A,S,F,E			1.81	A,S,R,F,E
	LEPTIN	**2.98**	S,F,E,C			**2.33**	A,S,R,F,E,C

The mean maximum LOD score of ten independent runs is shown for each locus. QTLs that obtained suggestive linkage (LOD ≥1.9) are indicated in bold. LOD < 1.5 are not shown. [a] Significant covariates (p-value <0.1) included in the best polygenic model are listed next to the LOD score: A=age, S=sex, R=polity of residence, F=farm work, E=education and C=cigarette smoker.

Table S4: Heritability estimates (h^2) of investigated traits and variance explained by covariates when interactions between covariates are taken into account.

Trait	h^2 (s.e.) (screened for Basic Set with interaction)[a]	Variance[b] (%)	h^2 (s.e.) (screened for Environmental Set with interaction)[a]	Variance[b] (%)
ABDCIR	0.57 (0.06) – A,R,A^2,A•R,S•R	30	0.56 (0.06) - A,R,A^2,A•R,F,S•F,A•E	31
ADIPONEC	0.47 (0.07) – S,R,A^2,A^2•S	19	0.48 (0.07) - S,R,A^2,A^2•S,F,S•F	19
LEPTIN	0.53 (0.06) – A,S,R,A•S,A•R,S•R,A^2•R	54	0.42 (0.07) – A,S,R,A^2•S,A•S,A•R,A^2•R, A^2•S•R,F,E,C,A•C	58
BMI	0.58 (0.06) – A,S,R,A^2,A•R	24	0.53 (0.08) - A,S,R,A•R,A^2,F,C,S•C,A•C,A•E	27
%BF	0.57 (0.07) – A,S,R,A^2,A•R,A^2•R	45	0.50[c] - A,S,R,F,A•R,A^2•R,A^2,C,A•C,S•F, S•C,A•E,S•E	49

h^2 for all traits are significantly different from zero (p-value $<10^{-5}$). [a] Significant covariates (p-value <0.1) included in polygenic model are presented: A=age, S=sex, R=polity of residence, F=farm work, E=education and C=cigarette smoker. [b] Variance explained by included covariates. [c] SOLAR failed to calculate s.e.

Table S5: Summary of multipoint LOD score ≥1.9 when using covariates from the Basic Set and the Environmental Set with and without interaction.

Cytogenetic position	Trait	Closest marker/s	LOD score (Basic Set with interaction)	LOD score (Basic Set from table 2)	LOD score (Environmental Set with interaction)	LOD score (Environmental Set from table 2)
1p21.2	BMI	D1S206			**1.98**	
2p11.2	ABDCIR	D2S2216	1.56		**2.16**	
9p22	ABDCIR	D9S157	**2.75**	**2.04**	**2.57**	**2.14**
"	%BF	D9S285	**2.70**	**2.30**		**2.48**
12q24.32	%BF	D12S324/D12S1659	1.72		**2.67**	
14q24.3	%BF	D14S74			**1.91**	
16q21-q23.1	BMI	D16S503/D16S515			**2.52**	
"	%BF	D16S503/D16S515			**2.65**	
"	LEPTIN	D16S503			**2.49**	**2.33**
"	ABDCIR	D16S503/D16S515	1.52	1.74	**2.16**	1.81

[a] For comparison LOD score ≥1.5 and <1.9 are shown if other model with interaction detected suggestive linkage (LOD score ≥1.9). Suggestive LOD scores are indicated in bold.

Figure S1

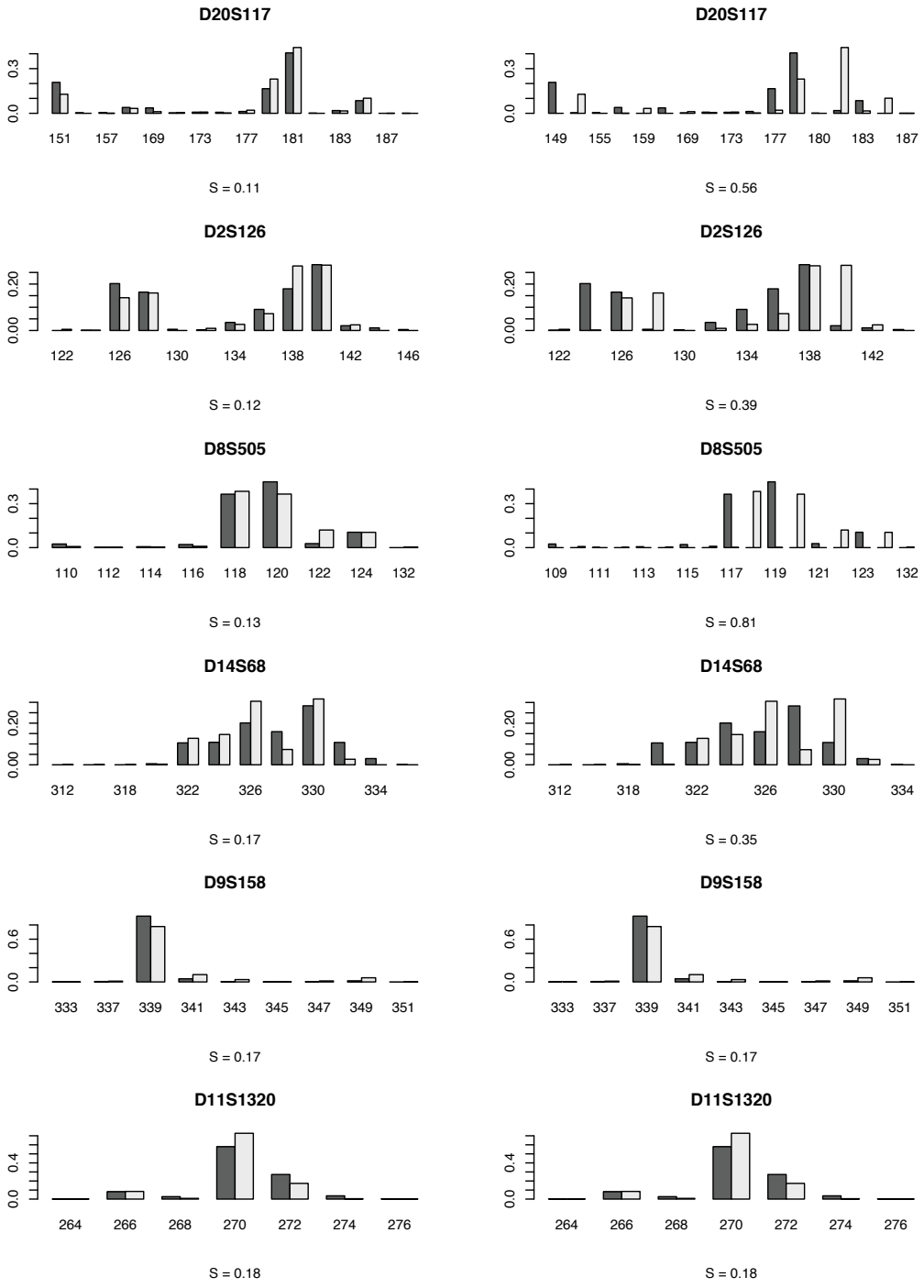
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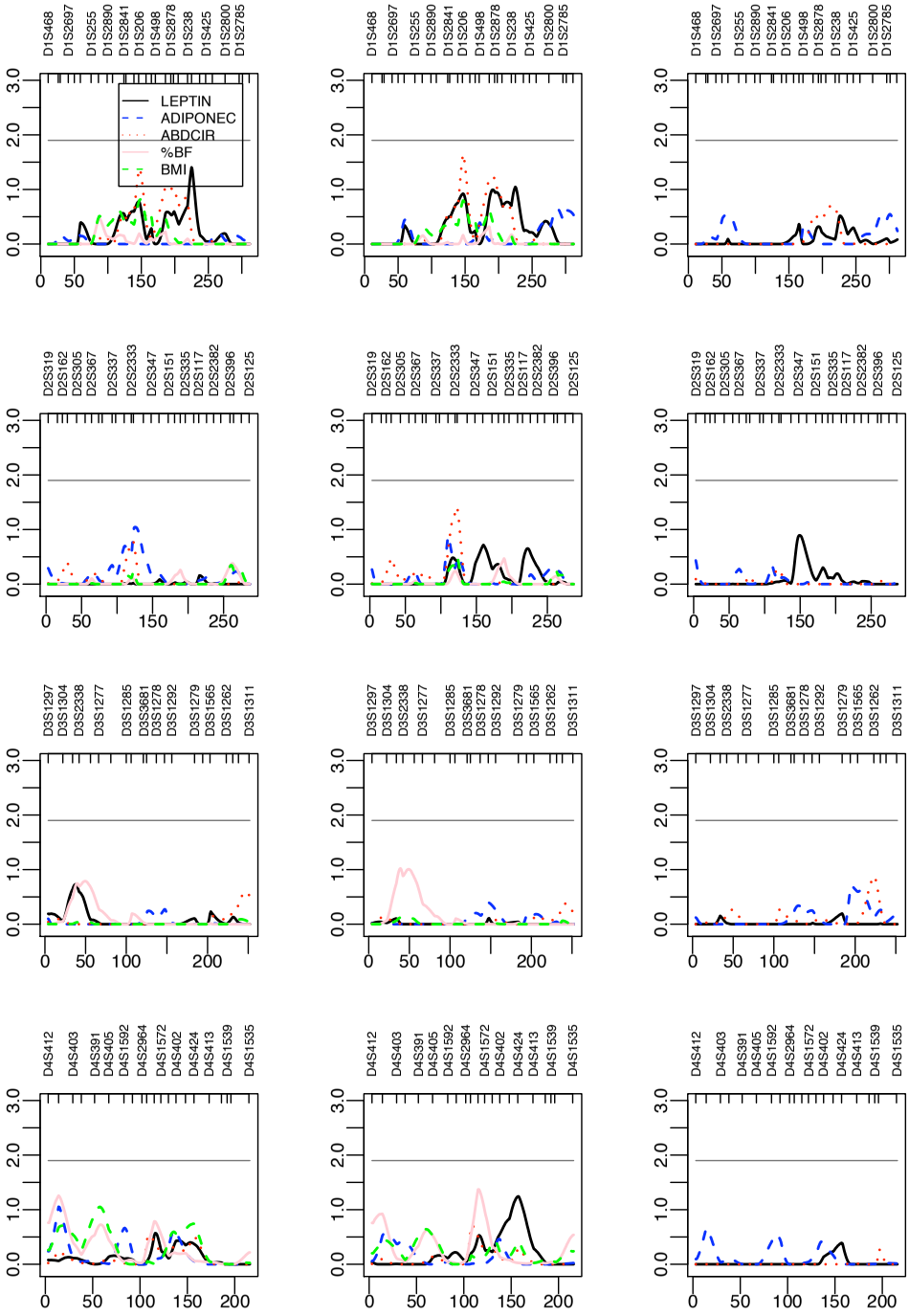
Figure S2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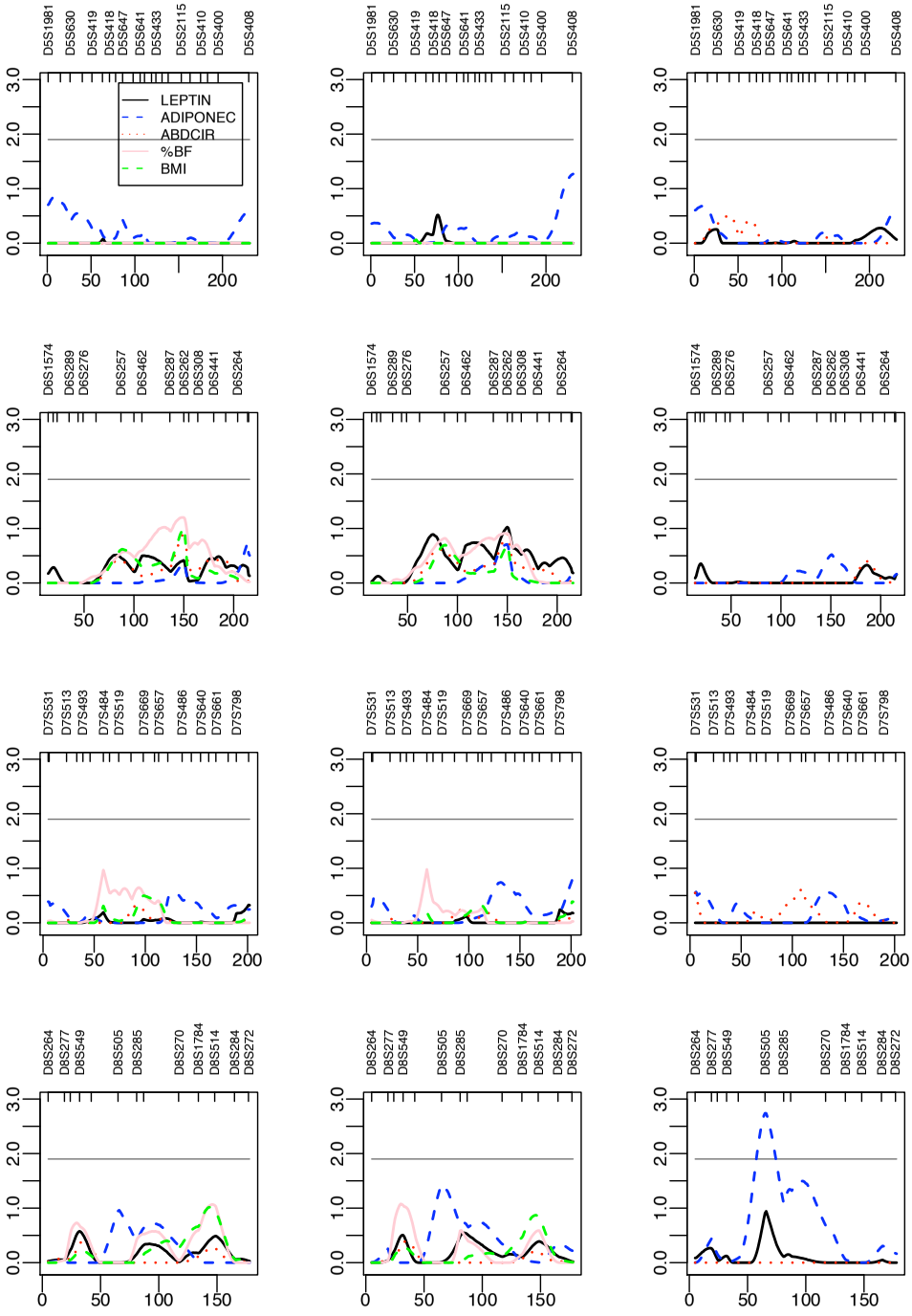
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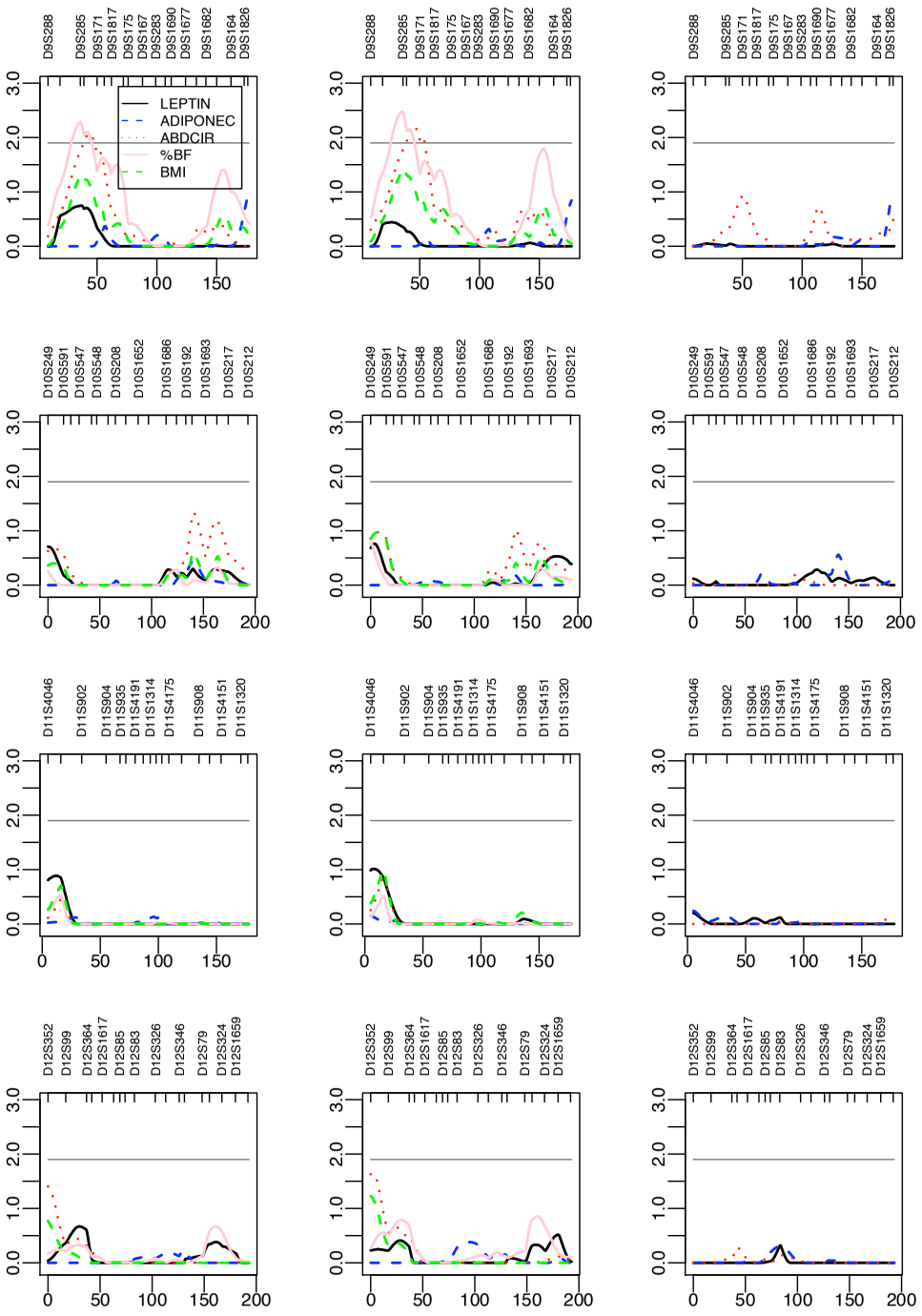
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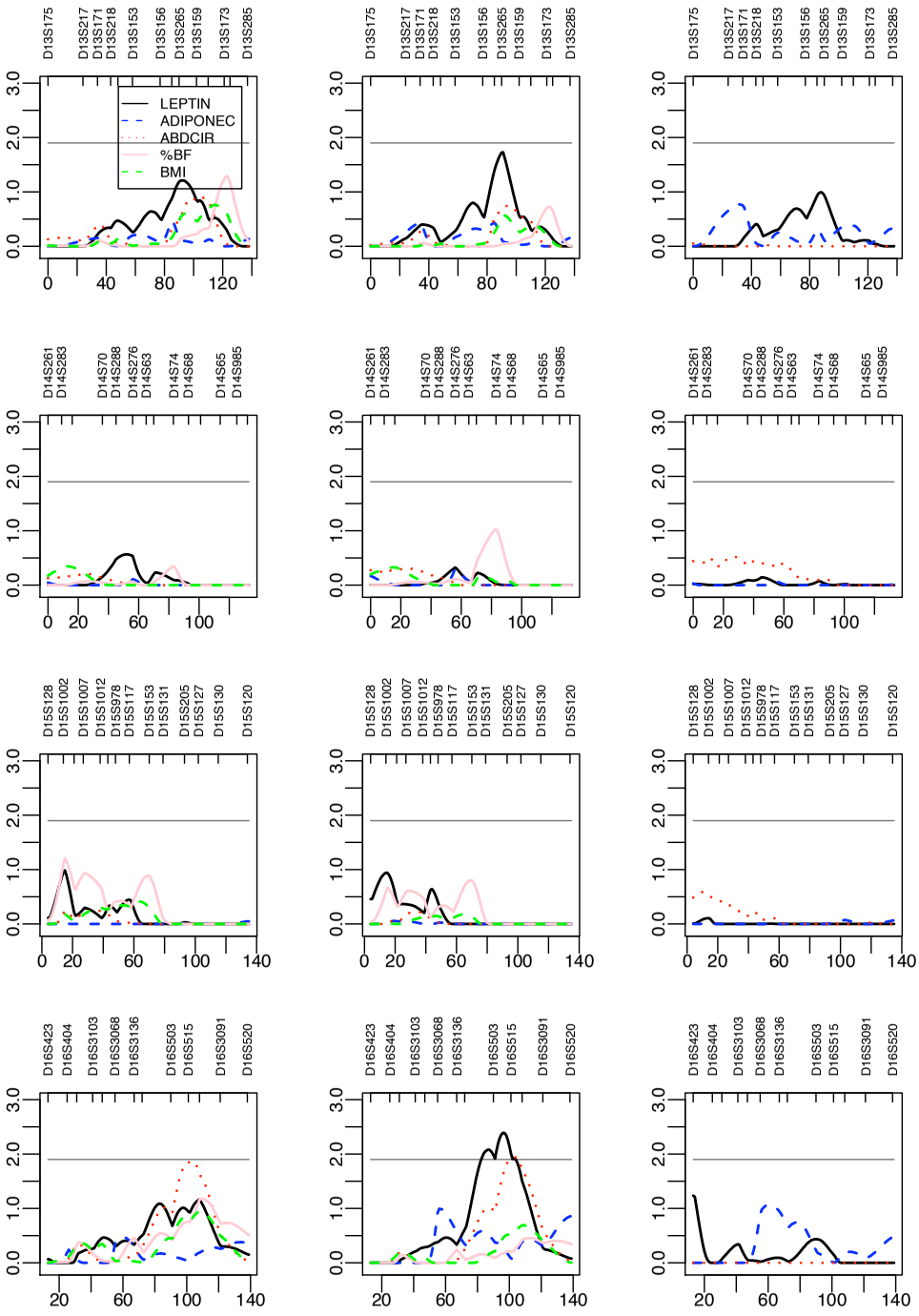
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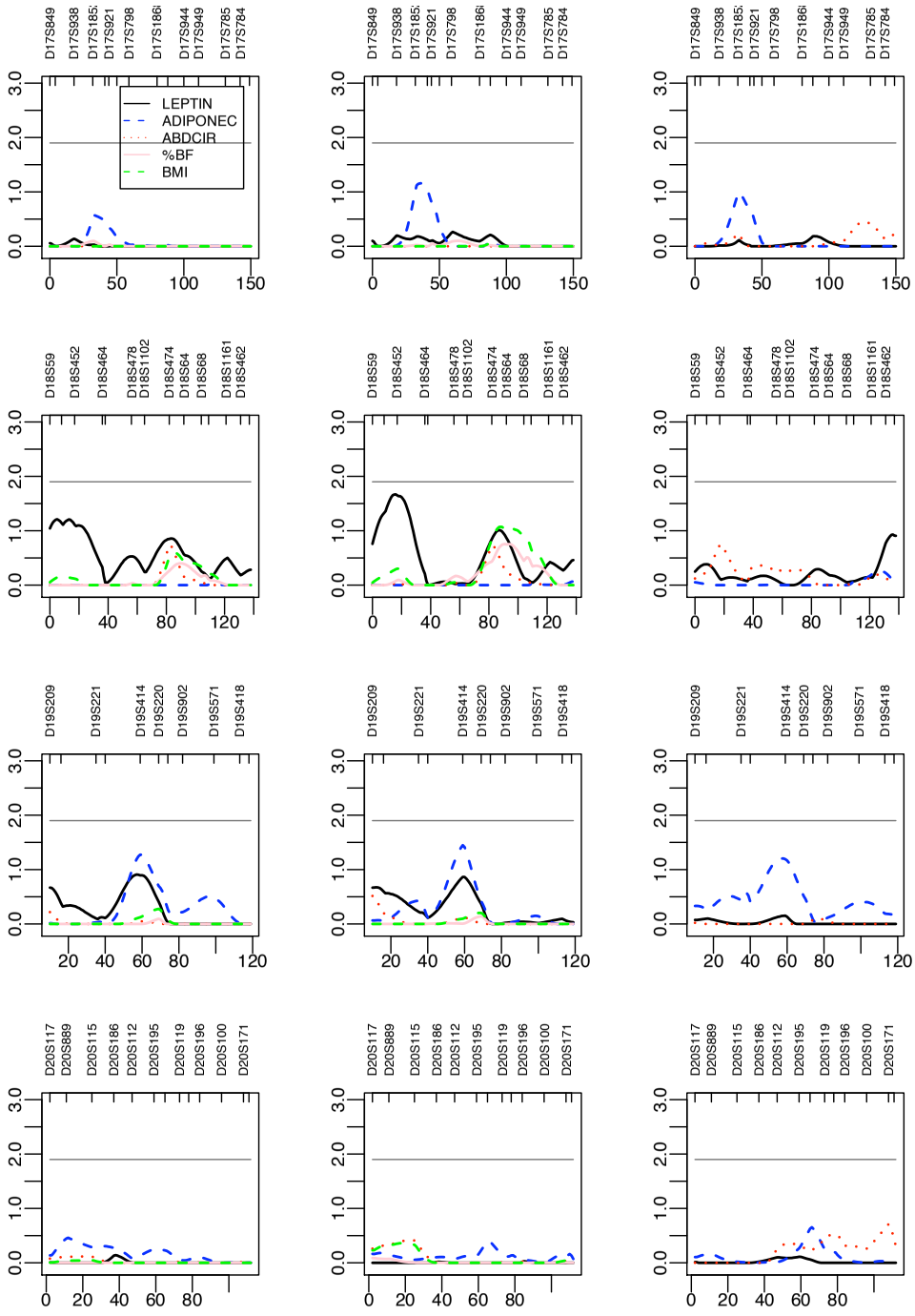
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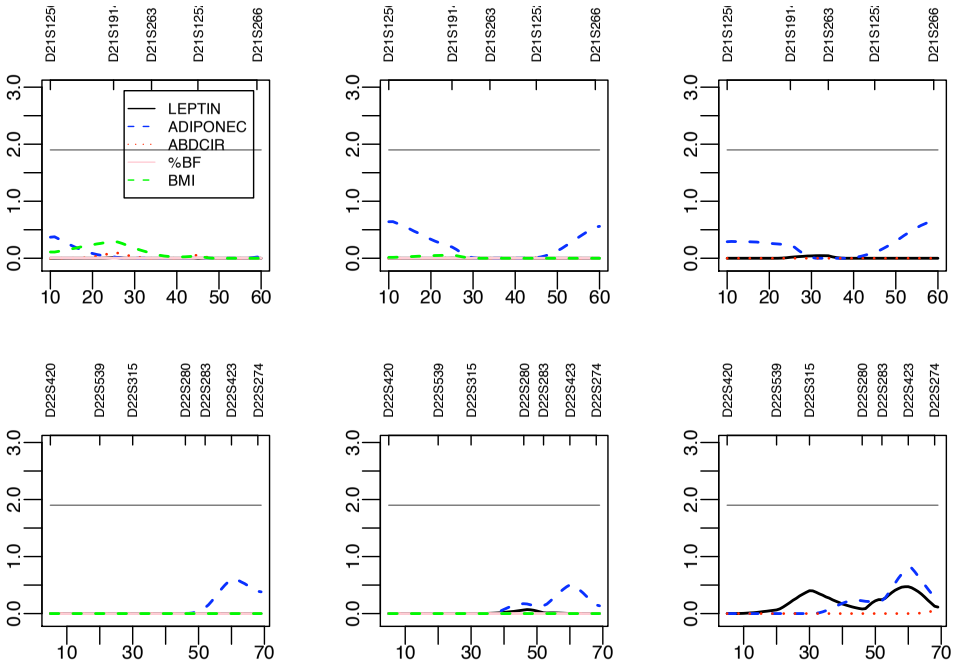
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ADIPONEC
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D22S274

Figure S3a

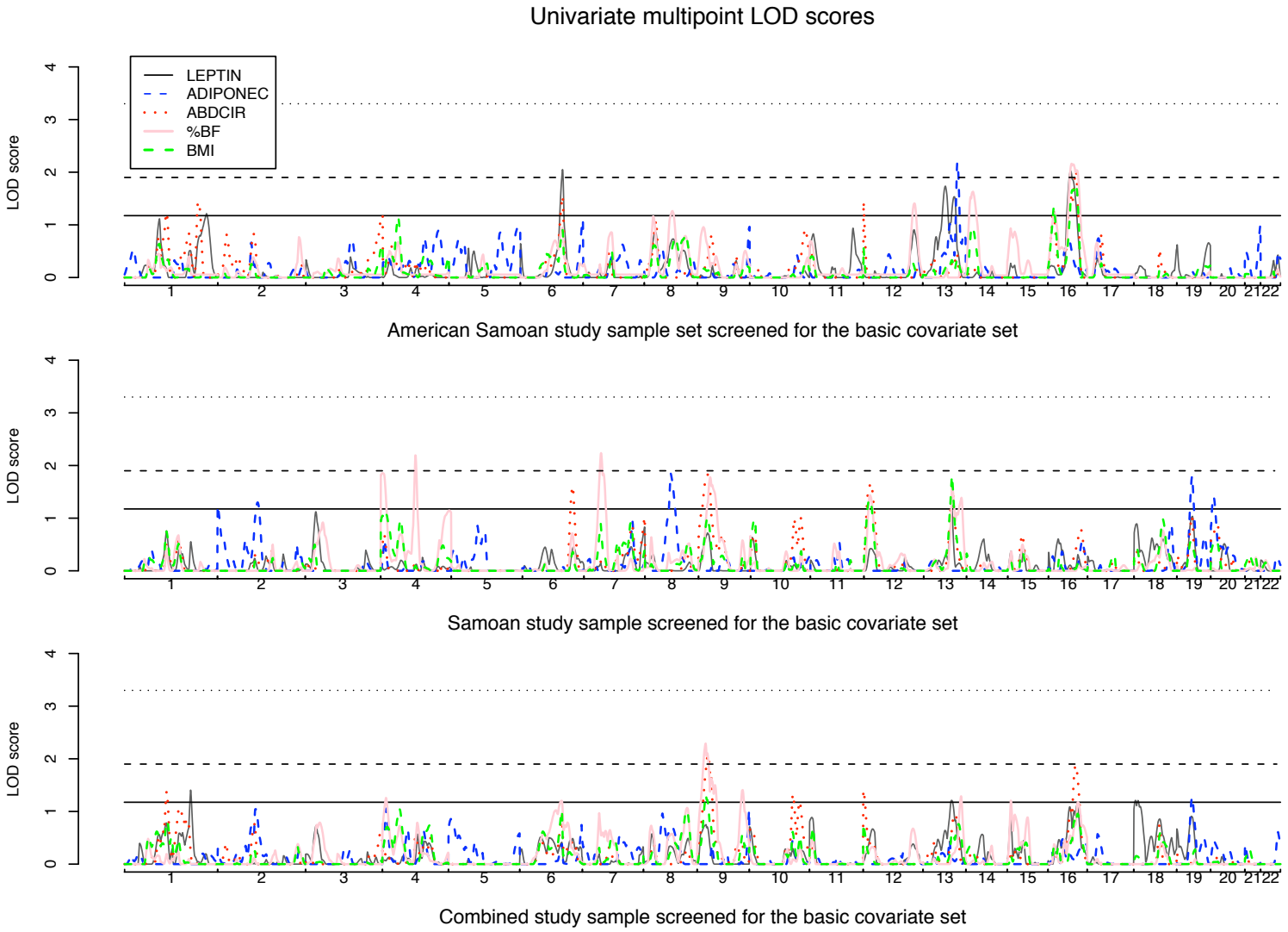
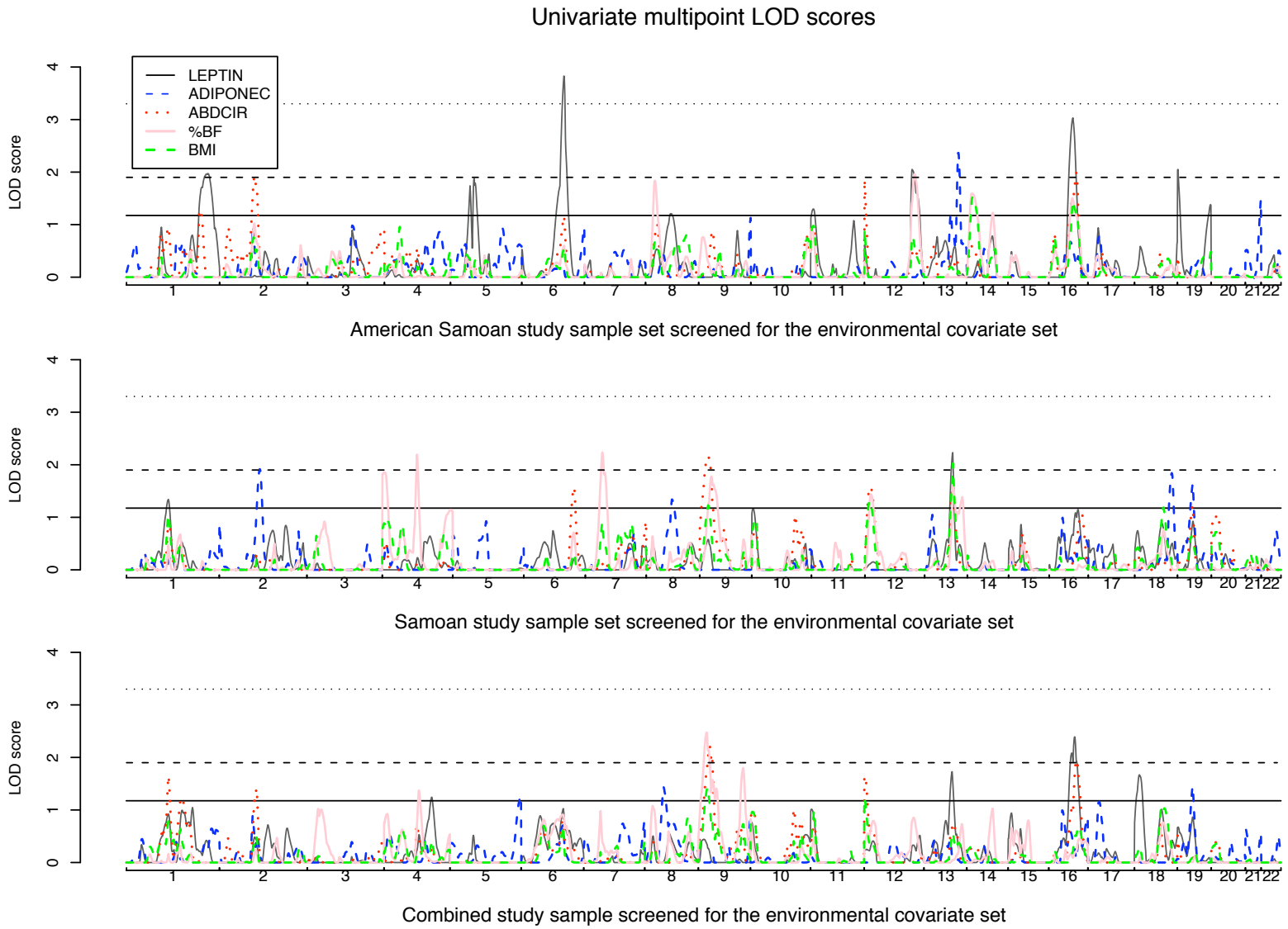

Univariate multipoint LOD scores
LEPTIN
ADIPONEC
ABDCIR
%BF
BMI
LOD score
0
1
2
3
4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
American Samoan study sample set screened for the basic covariate set
LOD score
Samoan study sample screened for the basic covariate set
LOD score
Combined study sample screened for the basic covariate set

Figure S3b


Univariate multipoint LOD scores
LEPTIN
ADIPONEC
ABDCIR
%BF
BMI
LOD score
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2
3
4
1
2
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4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
American Samoan study sample set screened for the environmental covariate set
Samoan study sample set screened for the environmental covariate set
Combined study sample screened for the environmental covariate set