

Exposure to non-persistent pesticides, BDNF, and behavioral function in adolescents: Exploring a novel effect biomarker approach

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Table S1. Urinary metabolites of non-persistent pesticides: classification, abbreviation, possible parent compound, and expiration of approval in the EU.

Classification	Urinary metabolites	Abbreviation	Possible parent compounds ^a				
			Approved in the EU ^b	Expiration of approval (year)	Not approved in the EU ^b		
Organophosphate insecticides	Non-specific metabolites	Diethyl thiophosphate	DETP	Chlorpyrifos-ethyl	2020	Chlorethoxyphos, coumaphos, diazinon, disulfoton, ethion, parathion, phorate, phosalone, phoxim, sulfotep, terbufos	
		Diethyl dithiophosphate	DEDTP				
	Specific metabolites	3,5,6-trichloro-2-pyridinol	TCPy	Chlorpyrifos-ethyl, chlorpyrifos-methyl, triclopyr	2020, 2020, 2019		-
		2-isopropyl-4-methyl-6-hydroxypyrimidine	IMPy	-	-		Diazinon
		Malathion dicarboxylic acid	MDA	Malathion	2022		-
Pyrethroid insecticides	Non-specific metabolites	3-Phenoxybenzoic acid	3-PBA	Alpha-cypermethrin, deltamethrin, beta-cyfluthrin, lambda-cyhalothrin	2019, 2019, 2019, 2023	Cyhalothrin, cyfluthrin, fenpropathrin, permethrin, tralomethrin	
	Specific metabolites	2,2-dichlorovinyl-2,2-dimethylcyclopropane-1-carboxylic acid	DCCA	Alpha-cypermethrin, beta-cyfluthrin	2019, 2019	Permethrin	
Carbamates	Specific metabolite	1-Naphthol	1-N	-	-	Carbaryl	
Dithiocarbamate fungicides	Non-specific metabolite	Ethylene thiourea	ETU	-	-	Mancozeb	

Table S2. Distribution of LOD-LOQ values of pesticide metabolites and CBCL score outcomes in adolescent males from the INMA-Granada cohort (n=140)

Pesticides	IMPy	MDA	TCPy	DETP	DEDTP	DCCA	3-PBA	1-N	ETU
LOD (ng/mL)	0.117	0.052	0.039	0.116	0.142	0.055	0.117	0.156	0.072
LOQ (ng/mL)	0.391	0.172	0.130	0.387	0.474	0.184	0.389	0.527	0.241
Q-SRM	151.09	273.0	197.91	169.01	184.98	113.00	213.05	143.04	103.03
tR (min)	1.74	6.38	3.21	3.14	2.29	2.61	3.36	1.45	1.73
a	-0.80231	3.2986	-0.76826	-1.22351	-1.53302	4.7924	-0.58052	-1.06224	-2.43736
b	0.60619	0.9203	0.91352	0.84444	0.84401	1.2798	0.83928	0.88090	0.97101
R ²	0.994	0.996	0.997	0.996	0.996	0.999	0.994	0.997	0.999
Mean accuracy (%)	92.77	98.64	88.29	101.98	99.46	90.49	93.58	101.68	94.64
% RSD	10.99	11.51	13.03	14.74	8.58	10.47	14.19	9.11	13.28
Bisulfite-converted primers used for PCR amplification									
Gene	Forward Primer				Reverse Primer				
<i>BDNF</i>	GATTTTGGTAATTAGTGTATTAGAGT GTT				/5Biosg/ATCAACCAAAAACCTCCATTTAATCTC				
Adolescents' behavioral functioning									
CBCL	Score range	Mean of scores ^a		SD	Median	n (%) in borderline or clinical range ^b			
Syndrome scores									
Anxious depressed	50-79	55.1		6.1	54.0	37 (25.7)			
Withdrawn	50-89	57.5		7.3	54.0	50 (34.7)			
Somatic complaints	50-79	58.1		7.2	58.0	53 (36.8)			
Social problems	50-79	55.1		6.4	51.0	33 (22.9)			
Thought problems	50-82	54.9		6.0	51.0	23 (15.1)			
Attention problems	50-86	56.1		7.0	53.0	31 (20.4)			
Rule-breaking behavior	50-89	54.0		6.2	51.0	22 (15.3)			
Aggressive behavior	50-81	55.2		6.4	52.0	33 (21.7)			
Composite scores									
Internalizing problems	34-79	55.6		9.2	55.0	46 (31.9)			
Externalizing problems	34-79	51.0		9.2	51.0	23 (16.0)			
Total problems	31-84	54.0		9.2	54.0	38 (26.4)			

^aFor all scales, raw scores were transformed to T-scores to compare each subject to a normative sample of adolescents.

BDNF= Brain-derived neurotrophic factor; IMPy= 2-isopropyl-4-methyl-6-hydroxypyrimidine; MDA= malathion dicarboxylic acid; TCPy= 3,5,6-trichloro-2-pyridinol; DETP= diethyl thiophosphate; DCCA= 2,2-dichlorovinyl-2,2-dimethylcyclopropane-1-carboxylic acid; 3-PBA= 3-phenoxybenzoic acid; 1-N= 1-naphthol; ETU= ethylene thiourea. LOD: Limit of detection; LOQ: Limit of quantification; Q-SRM: Selected reaction monitoring; tR: Retention time; RSD= Relative standard deviation; SD= standard deviation.

Table S3. Spearman's correlation among urinary pesticide metabolites (ng/mL).

		IMPy	MDA	TCPy	DETP	DCCA	3-PBA	1-N	ETU
IMPy	Rho	1.00	0.10	0.08	0.09	0.07	0.20	0.10	0.08
	p	.	0.25	0.31	0.27	0.37	0.02	0.22	0.34
MDA	Rho	0.10	1.00	0.11	0.15	-0.03	0.13	-0.03	0.04
	p	0.25	.	0.20	0.07	0.76	0.12	0.69	0.60
TCPy	Rho	0.08	0.11	1.00	0.06	0.08	0.17	0.07	0.00
	p	0.31	0.20	.	0.44	0.32	0.04	0.42	0.99
DETP	Rho	0.09	0.15	0.06	1.00	0.20	0.09	0.21	0.08
	p	0.27	0.07	0.44	.	0.02	0.29	0.01	0.31
DCCA	Rho	0.07	-0.03	0.08	0.20	1.00	-0.02	-0.01	0.03
	p	0.37	0.76	0.32	0.02	.	0.86	0.90	0.72
3-PBA	Rho	0.20	0.13	0.17	0.09	-0.02	1.00	0.04	0.04
	p	0.02	0.12	0.04	0.29	0.86	.	0.66	0.65
1-N	Rho	0.10	-0.03	0.07	0.21	0.08	0.04	1.00	0.03
	p	0.22	0.69	0.42	0.01	0.31	0.66	.	0.71
ETU	Rho	0.08	0.04	0.00	0.08	0.03	0.04	0.03	1.00
	p	0.34	0.60	0.99	0.31	0.72	0.65	0.71	.

IMPy= 2-isopropyl-4-methyl-6-hydroxypyrimidine; MDA= malathion dicarboxylic acid; TCPy=3,5,6-trichloro-2-pyridinol; DETP= diethyl thiophosphate; DCCA= 2,2-dichlorovinyl-2,2-dimethylcyclopropane-1-carboxylic acid; 3-PBA= 3-phenoxybenzoic acid; 1-N= 1-naphthol; ETU= ethylene thiourea.

Table S4. Beta regression coefficients (β , 95% CI) for adjusted associations between urinary pesticide metabolite concentrations and CBCL typical behavior score outcomes (n= 140).

	Syndrome scores								Composite scores		
	Anxious depressed	Withdrawn	Somatic complaints	Social problems	Thought problems	Attention problems	Rule-breaking behavior	Aggressive behavior	Internalizing problems	Externalizing problems	Total problems
IMPy^a	0.35 (-0.49,1.20)	0.76 (-0.24,1.75)	-0.38 (-1.38,0.63)	0.86 (0.00,1.71)*	0.56 (-0.27,1.39)	0.71 (-0.24,1.66)	1.31 (0.46,2.16)**	1.04 (0.19,1.90)*	0.31 (-0.97,1.60)	1.67 (0.43,2.92)**	1.40 (0.16,2.64)*
MDA^a	0.68 (-0.51,1.87)	0.22 (-1.19,1.63)	-0.41 (-1.83,1.01)	0.09 (-1.14,1.31)	0.14 (-1.03,1.32)	0.11 (-1.24,1.45)	0.26 (-0.98,1.49)	-0.06 (-1.29,1.18)	0.41 (-1.40,2.22)	0.12 (-1.68,1.91)	0.49 (-1.29,2.26)
DETP^b	0.33 (-0.54,1.19)	-0.88 (-1.89,0.12)†	0.46 (-0.56,1.48)	-0.21 (-1.09,0.68)	0.09 (-0.76,0.94)	0.11 (-0.86,1.09)	0.24 (-0.65,1.13)	-0.21 (-1.10,0.68)	0.03 (-1.28,1.34)	-0.14 (-1.44,1.16)	-0.11 (-1.39,1.18)
DCCA^a	-0.20 (-0.92,0.52)	-0.23 (-1.08,0.62)	0.21 (-0.65,1.06)	0.49 (-0.25,1.22)	0.32 (-0.39,1.02)	0.36 (-0.45,1.17)	0.42 (-0.33,1.16)	0.40 (-0.34,1.15)	0.08 (-1.02,1.17)	0.66 (-0.41,1.74)	0.54 (-0.53,1.61)
ETU^a	-0.73 (-1.50,0.04)†	0.07 (-0.85,0.99)	-0.36 (-1.28,0.56)	0.26 (-0.54,1.06)	-0.08 (-0.85,0.69)	0.03 (-0.85,0.91)	-0.17 (-0.98,0.63)	-0.04 (-0.85,0.77)	-0.89 (-2.06,0.29)	-0.41 (-1.58,0.76)	-0.57 (-1.72,0.59)
ΣOPs	0.69 (-0.53,1.91)	1.14 (-0.29,2.57)	-0.42 (-1.87,1.03)	1.36 (0.13,2.59)*	0.91 (-0.29,2.10)	1.02 (-0.35,2.38)	1.79 (0.56,3.01)**	1.19 (-0.05,2.44)†	0.75 (- 1.10,2.60)	2.06 (0.26,3.86)*	1.96 (0.18,3.75)*
ΣPYR	-0.37 (-1.25,0.51)	-0.35 (-1.38,0.69)	0.01 (-1.03,1.06)	0.49 (-0.40,1.39)	0.31 (-0.55,1.18)	0.31 (-0.68,1.30)	0.48 (-0.42,1.38)	0.48 (-0.42,1.38)	-0.15 (-1.49,1.18)	0.78 (-0.53,2.09)	0.47 (-0.83,1.78)

IMPy= 2-isopropyl-4-methyl-6-hydroxypyrimidine; MDA= malathion dicarboxylic acid; DETP= diethyl thiophosphate; ΣOPs= sum of organophosphate metabolites (IMPy+MDA+TCPy); DCCA= 2,2-dichlorovinyl-2,2-dimethylcyclopropane-1-carboxylic acid; ΣPYR= sum of pyrethroid metabolites (3-PBA+DCCA); ETU= ethylene thiourea. Regression estimates reflect average change in behavioral score for each log-unit increment in urinary metabolite concentration (a) or detected *versus* undetected concentrations (b). For all scales, raw scores were transformed to T-scores to compare each subject to a normative sample of adolescents.

Models were adjusted for adolescents' age (months), BMI, alcohol consumption (>1 beverage/month/<1 beverage/month), season of urine collection (spring/summer/autumn/winter), urinary creatinine (mg/dL), and maternal education (primary/secondary/university). *p<0.05; **p<0.01; †p<0.1

Table S5. Weighted Quantile Sum (WQS) model to assess the mixture effect of urinary IMPy, MDA, DCCA, and ETU on adolescents' behavior (n=140), serum BDNF protein levels (n=130), and BDNF DNA methylation (n=118).

CBCL scoring	WQS	
	β (SE)	p-value
Syndrome scores		
Anxious depressed	0.84 (0.98)	0.39
Withdrawn	2.71 (1.38)	0.05
Somatic complaints	0.43 (1.31)	0.75
Social problems	4.27 (1.33)	0.002
Thought problems	2.24 (1.34)	0.10
Attention problems	1.81 (1.48)	0.22
Rule-breaking behavior	2.10 (1.28)	0.11
Aggressive behavior	2.15 (1.40)	0.12
Composite scores		
Internalizing problems	0.76 (1.63)	0.64
Externalizing problems	3.03 (2.01)	0.14
Total problems	2.81 (1.88)	0.14
BDNF biomarkers	WQS	
	β (SE)	p-value
Serum BDNF	-1.98 (2.10)	0.35
CpG1	0.18 (0.17)	0.30
CpG2	0.07 (0.07)	0.34
CpG3	0.26 (0.12)	0.03
CpG4	0.41 (0.25)	0.11
CpG5	0.25 (0.16)	0.12
CpG6	0.49 (0.27)	0.07
CpGt	0.27 (0.12)	0.03

BDNF= Brain-derived neurotrophic factor; SE= Standard error; ^aFor all scales, raw scores were transformed to T-scores to compare each subject to a normative sample of adolescents. Higher scoring indicated worse behavioral outcomes. Models were adjusted for adolescents' age (months), BMI, alcohol consumption (>1 beverage/month/<1 beverage/month), season of urine collection (spring/summer/autumn/winter), creatinine concentrations (mg/dL), and maternal education (primary/secondary/university).

Table S6. Beta regression coefficients (β , 95% CI) for adjusted associations between urinary pesticide metabolite concentrations and serum BDNF protein levels (n=130) and DNA methylation of the BDNF gene (n=118).

	Serum BDNF	CpG 1	CpG 2	CpG 3	CpG 4	CpG 5	CpG 6	CpG total
IMPy	-1.16 (-2.53,0.22)†	0.02 (-0.10,0.14)	0.04 (-0.04,0.11)	0.05 (-0.04,0.14)	0.01 (-0.19,0.21)	0.05 (-0.07,0.18)	0.08 (-0.09,0.26)	0.04 (-0.05,0.14)
MDA	-1.86 (-3.81,0.10)†	0.17 (0.00,0.33)*	0.10 (-0.01,0.20)†	0.10 (-0.03,0.22)	0.04 (-0.23,0.31)	0.06 (-0.11,0.23)	-0.03 (-0.27,0.22)	0.07 (-0.06,0.20)
DETP	-3.59 (-7.60,0.29)†	-0.08 (-0.42,0.25)	0.09 (-0.12,0.31)	0.12 (-0.13,0.38)	0.21 (-0.35,0.76)	0.05 (-0.30,0.40)	0.34 (-0.16,0.83)	0.12 (-0.15,0.39)
DCCA	0.10 (-1.06,1.26)	0.04 (-0.06,0.14)	-0.01 (-0.08,0.05)	-0.01 (-0.08,0.07)	-0.05 (-0.21,0.12)	-0.02 (-0.13,0.08)	-0.02 (-0.17,0.13)	-0.01 (-0.09,0.07)
ETU	-0.85 (-2.06,0.36)	0.03 (-0.07,0.14)	0.06 (-0.01,0.13)†	0.10 (0.03,0.18)**	0.14 (-0.03,0.31)	0.06 (-0.05,0.17)	0.13 (-0.03,0.28)	0.09 (0.01,0.17)*
ΣOPs	-2.37 (-4.33,-0.40)*	0.09 (-0.07,0.26)	0.10 (-0.01,0.20)†	0.09 (-0.03,0.22)	0.04 (-0.23,0.32)	0.12 (-0.05,0.29)	0.11 (-0.14,0.35)	0.09 (-0.40, 0.23)
ΣPYR	-0.13 (-1.55,1.29)	0.06 (-0.06,0.18)	-0.01 (-0.09,0.06)	-0.01 (-0.10,0.08)	-0.04 (-0.24,0.16)	-0.02 (-0.14,0.11)	-0.01 (-0.19,0.17)	-0.00 (-0.10,0.09)

BDNF= Brain-derived neurotrophic factor; IMPy= 2-isopropyl-4-methyl-6-hydroxypyrimidine; MDA= malathion dicarboxylic acid; DETP= diethyl thiophosphate; ΣOPs= sum of organophosphate metabolites (IMPy+MDA+TCPy); DCCA= 2,2-dichlorovinyl-2,2-dimethylcyclopropane-1-carboxylic acid; 3-PBA= 3-phenoxybenzoic acid; ΣPYR= sum of pyrethroid metabolites (3-PBA+DCCA); 1-N= 1-naphthol; ETU= ethylene thiourea.

Regression estimates reflect average change in BDNF serum level or gene methylation for each log-unit increment in urinary metabolite concentration (a) or detected *versus* undetected concentrations (b). For all scales, raw scores were transformed to T-scores to compare each subject to a normative sample of adolescents. Models were adjusted for adolescents' age (months), BMI, alcohol consumption (>1 beverage/month/<1 beverage/month), season of urine collection (spring/summer/autumn/winter), urinary creatinine (mg/dL), and maternal education (primary/secondary/university).

*p<0.05; **p<0.01; †p<0.10

Table S7. Beta regression coefficients (β , 95% CI) for the association between serum BDNF protein levels and behavioral function in adolescent males (n= 130).

		Syndrome scores								Composite scores		
		Anxious depressed	Withdrawn	Somatic complaints	Social problems	Thought problems	Attention problems	Rule-breaking behavior	Aggressive behavior	Internalizing problems	Externalizing problems	Total problems
Serum BDNF protein level	Continuous	0.01 (-0.11,0.12)	-0.04 (-0.17,0.09)	-0.04 (-0.19,0.11)	-0.03 (-0.15,0.10)	-0.10 (-0.21,0.01)†	-0.05 (-0.18,0.08)	-0.08 (-0.19,0.04)	-0.03 (-0.14,0.09)	-0.05 (-0.24,0.14)	-0.04 (-0.21,0.14)	-0.06 (-0.24,0.12)
	T2 (28.3-36.7)	-0.12 (-2.74,2.50)	-2.10 (-5.11,0.92)	-1.33 (-4.78,2.12)	-1.95 (-4.72,0.83)	-2.93 (-5.47,-0.38)*	-1.53 (-4.57,1.50)	-2.71 (-5.39,-0.02)*	-1.89 (-4.58,0.79)	-3.00 (-7.37,1.37)	-2.87 (-6.99,1.26)	-3.57 (-7.72,0.58)†
	T3 (36.8-56.0)	0.60 (-2.10,2.30)	-1.71 (-4.82,1.40)	-0.95 (-4.51,2.60)	-1.03 (-3.89,1.82)	-3.03 (-5.65,-0.40)*	-0.23 (-3.36,2.89)	-2.35 (-5.11,0.42)†	-1.28 (-4.04,1.49)	-1.16 (-5.66,3.34)	-1.64 (-5.88,2.61)	-1.70 (-5.98,2.58)
	p-trend	0.65	0.71	0.61	0.49	0.03	0.90	0.10	0.37	0.63	0.46	0.46

^aFor all scales, raw scores were transformed to T-scores to compare each subject to a normative sample of adolescents. Higher scoring indicated worse behavioral outcomes. BDNF= Brain-derived neurotrophic factor.

Models were adjusted for the following covariates: adolescent's age, BMI, alcohol consumption, and maternal education. * $p \leq 0.05$; † $p < 0.10$

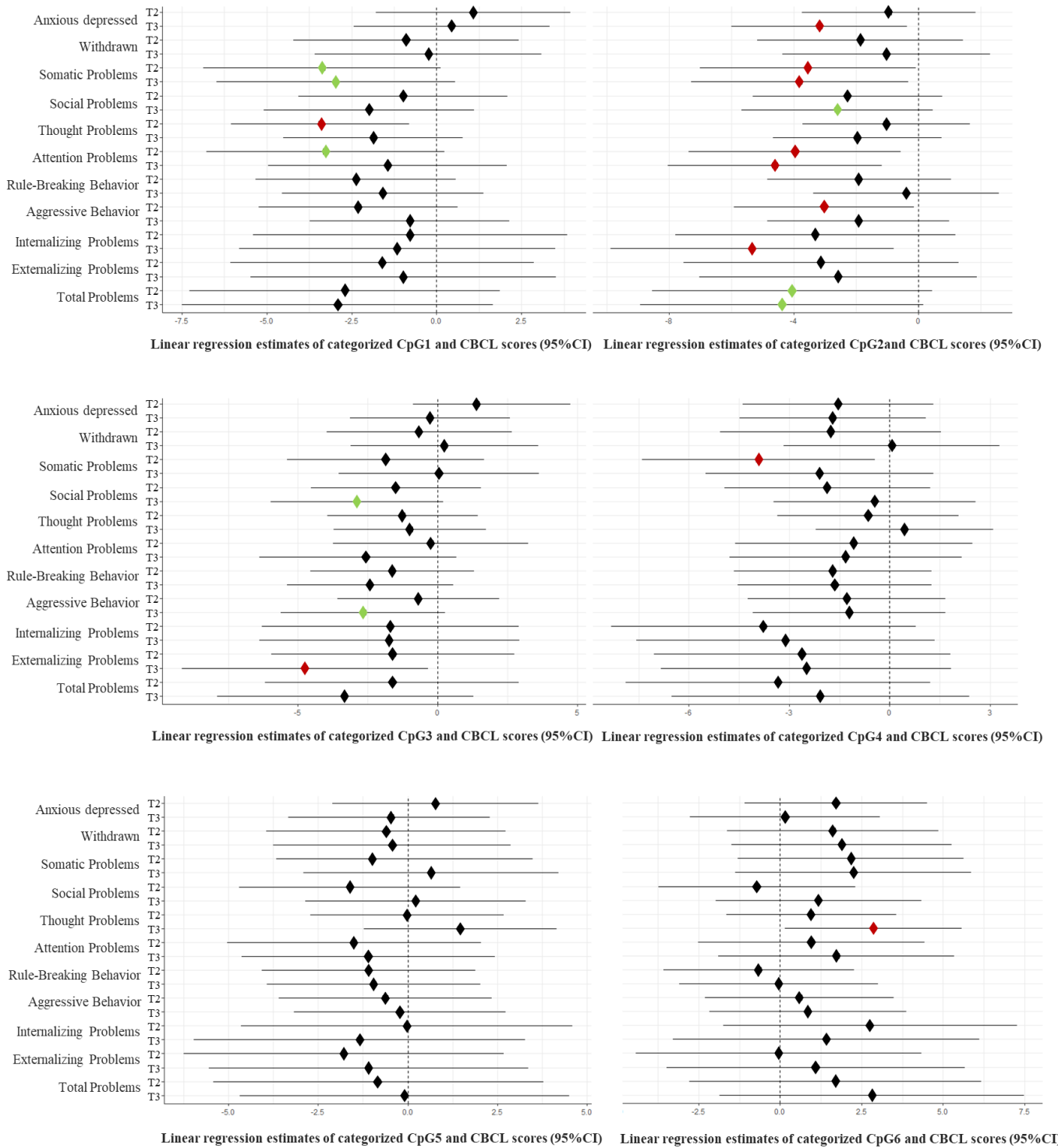


Figure S2. Forest plot showing the linear regression estimates (β , 95%CI) for the association between categorized BDNF gene DNA methylation percentages and adolescents' behavior (n=118). Model was adjusted for adolescent's age, BMI, alcohol consumption, season of urinary collection and maternal education. Red diamonds show statistical associations with p-values below 0.05; Green diamonds show p-values below 0.1; Black diamonds show p-values above 0.1.