SUPPLEMENTARY MATERIAL

Biomarker Changes Associated with both Dulaglutide and Cardiovascular Events in the REWIND Randomized Controlled Trial: A Nested Case-Control *post hoc* Analysis

Contents

Supplemental Description of Metabolite Assay Methods

Targeted metabolomics was performed on plasma samples collected after 2 years from patient enrollment. Plasma samples were frozen immediately after collection and thawed prior to analysis. Metabolomics data were acquired using a targeted liquid chromatography-mass spectrometry approach. Data were acquired using a Shimadzu Nexera X2 UHPLC system coupled to an AB SCIEX 6500+ triple quadrupole mass spectrometer equipped with an electrospray source. Two chromatographic methods were applied: one using a Waters Acquity BEH Amide 15 mm × 2.1 mm, 3.5 µm particle size, column (assay A), and another using a Waters XSelect HSS T3 C18 15 mm × 2.1 mm, 3.5 µm particle size, column (assay B). Both columns were maintained at +40°C. Elution solvents for both methods were 10 mM ammonium formate adjusted with 0.1% formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B) and run under gradient conditions. Data were acquired using scheduled multiple reaction monitoring (MRM) mode with polarity switching. A total of 250 polar metabolites were targeted. For most of the metabolites, identification was supported by one qualifier ion monitored in addition to a quantifier ion. To resolve interferences amongst specific metabolites, two qualifier ions were monitored (data not reported). Two extraction protocols were used for assay A and B, respectively. Frozen plasma samples were thawed in an ice bath. For assay A, 150 µL of pre-chilled acetonitrile-methanol (1:1 v/v) solvent mixture was added to 25-µL plasma aliquot. For assay B, 150 µL of pre-chilled methanol-water (4:1 v/v) solvent mixture was added to a second 25-µL plasma aliquot. Samples were mixed thoroughly and incubated at −20°C overnight. Both extraction solutions were spiked with a mix of stable labeled internal standards. The samples were then centrifuged for 15 min at 14000 g at +4°C, and the supernatants were removed for LC-MS/MS analysis. For each assay, a pooled calibrator sample was created for each method by combining an aliquot of the same volume from all the extracted samples together. The pooled calibrator (100% calibration point) was serially diluted in the same extraction solvent and used to create a calibration curve (75%, 60%, 40%, 25%, 10%) for relative quantitation and batch-to-batch data normalization. A pooled QC sample was also prepared at 50% (1:1 v/v dilution). Individual samples were further diluted 1:1 (v/v) with extraction solution, transferred into a 96-well plate and injected for LC-MS/MS analysis. Only analytes detected at the lowest pooled calibrator with signal-to-noise > 3 and detected in more than 75% of the individual samples were quantified. Relative quantitation to the pooled calibrators was achieved using a log-log linear regression model. Relative values extrapolated outside the calibration curve were not imputed. Peak areas were integrated using the AB SCIEX MultiQuant 3.0.2 software. Metabolite areas were normalized to matched internal standard area responses. For analytes with no matching stable labeled internal standards, the optimal match (picked amongst the internal standards monitored under the same polarity and within the same assay) was the one giving the minimum relative standard deviation of the pooled calibrator responses and best linearity fit. For statistical analysis metabolites showing coefficient of variations (CV%) below 20% for the qualifier-to-quantifier MRM ratio, as well as linear regression coefficients (R2) above 0.85 were reported.

Table S1: List of Measured Proteins and the Assay Systems Used for Analyses	
Protein Biomarker	Assay System
High sensitivity C-reactive Protein	Roche Cobas
Growth Differentiation Factor 15	R&D Systems ELISA
C-Peptide	Roche Cobas
NT-proBNP	MSD V-plex
Intact Proinsulin	Invitron ELISA
Insulin like growth factor binding protein 2	R&D Systems ELISA
Serum amyloid A	MSD V-plex Vascular Inflammation panel
Apolipoprotein B	Roche Cobas
Apolipoprotein CIII	Roche Cobas
Interleukin 6	MSD V-plex proinflammatory panel
Cytokeratin 18	Diapharma ELISA
Interferon gamma	MSD V-plex proinflammatory panel
Soluble intercellular adhesion molecule 1	MSD V-plex Vascular Inflammation panel
Soluble vascular cell adhesion molecule 1	MSD V-plex Vascular Inflammation panel
Interleukin 8	MSD V-plex proinflammatory panel
Interleukin 10	MSD V-plex proinflammatory panel
Apolipoprotein AI	Roche Cobas
Insulin	In house ELISA
Tumor necrosis factor alpha	MSD V-plex proinflammatory panel

Table S2: Coefficient of Variance (CV) and Lower Limit of Detection of Protein Biomarker Assays			
Protein Biomarker	**Lower Limit of Detection**	**Intra-assay %CV**	**Inter-assay %CV**
High sensitivity C-reactive Protein	0.15 mg/L	single sample analysis	<3 SD of Roche clinical-grade quality control
Growth Differentiation Factor 15	8.95 pg/mL	2.7%	10.4%
C-Peptide	0.01 ng/mL	single sample analysis	<3 SD of Roche clinical-grade quality control
NT-proBNP	1.52 pg/mL	6.0%	6.4%
Intact Proinsulin	3.6 pmol/L	18%	13%
Insulin like growth factor binding protein 2	14.3 ng/mL	8.0%	5.9%
Serum amyloid A	0.97 ng/mL	6.1%	11.3%
Apolipoprotein B	3 mg/dL	single sample analysis	<3 SD of Roche clinical-grade quality control
Apolipoprotein CIII	3 mg/dL	single sample analysis	<3 SD of Roche clinical-grade quality control
Cytokeratin 18	65.1 U/L	11.6%	13%
Interferon gamma	0.06 pg/mL	3.7%	3.6%
Interleukin 6	0.005 pg/mL	4.6%	5.9%
Soluble intercellular adhesion molecule 1	0.15 ng/mL	5.9%	5.3%
Soluble vascular cell adhesion molecule 1	0.65 ng/mL	4%	2.1%
Interleukin 8	0.01 pg/mL	4.5%	7.8%
Interleukin 10	0.01 pg/mL	9.96%	10.1%
Apolipoprotein AI	3 mg/dL	single sample analysis	<3 SD of Roche clinical-grade quality control
Insulin	6.1 pg/mL	6.3%	8.9%
Tumor necrosis factor alpha	0.05 pg/mL	4.2%	6.3%

SD – standard deviation

Table S3: List of Metabolite Biomarkers in the Analysis		
2-hydroxybutyric acid	Arginine	Beta-alanine
Threonine	Octanoylcarnitine	Hydroxyoctenoylcarnitine
3-hydroxybutyric acid	Hydroxytetradecenoylcarnitine	Arachidonoylcarnitine
Asparagine	Octenoylcarnitine	N-acetylaspartic acid
Guanidinoacetic acid	Tetradecenoylcarnitine	Isoleucine
Citrulline	N-acetylthreonine	Malic acid
Free carnitine	Tryptophan	Glutaric acid
Hydroxydecanoylcarnitine	Creatinine	Proline
Glutamine	Acetylcarnitine	Phenylpyruvic acid
Creatine	Serine	Kynurenine
Uridine monophosphate	Palmitoleoylcarnitine	Allantoin
Glycine	Nicotinamide	Decadienoylcarnitine
3-hydroxyisobutyric acid	N-acetylmethionine	2,3-pyridinedicarboxylic acid
3-phenyllactic acid	Octadecanoylcarnitine	Taurine
CMPF[1]	Linoleoylcarnitine	N-acetylserine
2-hydroxy-3-methylbutiric acid	Xanthurenic acid	Lysine
Hydroxydodecenoylcarnitine	Decanoylcarnitine	Sarcosine
Tyrosine	1-methyladenosine	S-adenosylhomocysteine
Butenoylcarnitine	Decatrienoylcarnitine	Glutamic acid
Dodecenoylcarnitine	Arachidoylcarnitine	N-acetyltryptophan
Ketoleucine	Urea	N-acetylalanine
Alpha-ketoisovaleric acid	Succinic acid	2-aminoadipic acid
Asymmetric dimethylarginine	N-alpha-acetyllysine	Pantothenic acid
Uridine	Palmityolcarnitine	Citric acid
Leucine	3-aminoisobutyric acid	Uric acid
Decenoylcarnitine	Heptanoylcarnitine	Isovalerylcarnitine
Ornithine	Methionine sulfoxide	Hypoxanthine
tetradecadienoylcarnitine	Betaine	Trans aconitic acid
2-aminobutyric acid	Myristoylcarnitine	C6:2 carnitine
Eicoseneoylcarnitine	N-acetylputrescine	Riboflavin
Lactic acid	3-methyl-2-oxovaleric acid	Propionylcarnitine
Trimethylamine-N-oxide	1-methylnicotinamide	Aspartic acid
N –alpha acetyl-L-arginine	N, N-dimethylglycine	Symmetric dimethylarginine
Hydroxyproline	Butyrylcarnitine	Inosine
Cholesterol sulfate	4-pyridoxic acid	Alanine
Pyroglutamic acid	Phenylalanine	1-methylhistidine
N-2-furoylglycine	2-hydroxyisocaproic acid	S-methylcysteine sulfoxide
N-acetylglutamic acid	C18:3 carnitine	Isocitric acid
Imidazole-4-acetic acid	Xanthine	Alpha-ketoglutaric acid
Cystine	Mevalonic acid	3-methylhistidine
Valine	Kynurenic acid	Histidine

Oleoylcarnitine	4-oxoproline_pipecolicacid	Guanosine
Hexadecadienoylcarnitine	N-epsilon-acetyllysine	Ethanolamine
N-acetylcitrulline	2,3-dihydroxybenzoic acid	S-methylcysteine
Homocitrulline	Dodecanoylcarnitine	N-acetylornithine

[1]CMPF - carboxy-4-methyl-5-propyl-2-furanpropanoic acid

Table S4: Association of Dulaglutide with the 2-Year Change in Protein Levels

Protein Biomarker	Beta Estimate (95%CI) for Dulaglutide	P*
High sensitivity C-reactive Protein	-0.203 (-0.297, -0.110)	0.000020*
Growth Differentiation Factor 15	-0.079 (-0.112, -0.045)	0.000006*
C-Peptide	0.135 (0.070, 0.200)	0.000050*
NT-proBNP	-0.145 (-0.229, -0.060)	0.000845*
Intact Proinsulin	-0.094 (-0.156, -0.031)	0.003511
Insulin like growth factor binding protein 2	0.049 (0.014, 0.084)	0.005720
Serum amyloid A	-0.131 (-0.236, -0.026)	0.014509
Apolipoprotein B	-0.022 (-0.043, 0.000)	0.051646
Apolipoprotein CIII	-0.047 (-0.100, 0.006)	0.083511
Cytokeratin 18	-0.046 (-0.100, 0.009)	0.103077
Interferon gamma	0.017 (-0.058, 0.093)	0.169657
Interleukin 6	-0.039 (-0.100, 0.022)	0.211904
Soluble intercellular adhesion molecule 1	-0.024 (-0.064, 0.015)	0.231563
Soluble vascular cell adhesion molecule 1	-0.024 (-0.065, 0.016)	0.231781
Interleukin 8	0.038 (-0.025, 0.101)	0.239601
Interleukin 10	0.036 (-0.032, 0.103)	0.304340
Apolipoprotein AI	0.003 (-0.009, 0.015)	0.576137
Insulin	-0.014 (-0.078, 0.050)	0.662862
Tumor necrosis factor alpha	-0.004 (-0.040, 0.032)	0.836807

The effect of dulaglutide on the 2-year difference of the natural logarithm (ln) levels of each protein (calculated as ln (2-year/baseline) adjusted for ln (baseline biomarker level), age, sex, white identification, systolic blood pressure, previous cardiovascular disease, current smoking, body mass index, HbA1c, eGFR, LDL cholesterol, and albuminuria is shown. The biomarkers are sorted from the lowest to highest P values for the beta coefficient, and negative values denote a greater fall in level from baseline to 2 years with dulaglutide versus placebo. *P values that are significant at a level of 0.05/19 = 0.0026.

Table S5: Sensitivity Analysis: Association Between MACE Status and Change in Specific Biomarker Levels from Baseline, After Excluding Cases that Occurred Before the 2-Year Visit (and their Controls)

		Adjusted for Dulaglutide and Risk Factors OR per Unit Higher Level (95%CI)	P
Proteins	High sensitivity CRP	1.207 (1.069, 1.362)	0.002
	NT-proBNP	1.128 (0.980, 1.297)	0.094
	GDF15	1.709 (1.208, 2.418)	0.002
	C-peptide	1.162 (0.984, 1.371)	0.076
Metabolites	2-hydroxybutyric Acid	1.146 (0.812, 1.619)	0.439
	Threonine	1.018 (0.511, 2.026)	0.960

OR – odds ratio; CI – confidence interval; The relationship between a 1-unit higher Ln(2-year) – Ln (baseline) is shown. Risk factors included in the model were age, sex, white self-identification, systolic blood pressure, previous cardiovascular disease, current smoking, body mass index, HbA1c, the estimated glomerular filtration rate (eGFR), LDL cholesterol, and albuminuria and (for the dulaglutide model) and the interaction of dulaglutide and the change in biomarker. The significance threshold for proteins is 0.05/4=0.0125.

Table S6: Association of Dulaglutide with the 2-Year Change in Metabolite Levels		
Metabolite Biomarker	**Beta Estimate (95%CI) for Dulaglutide**	**P***
2-hydroxybutyric acid	-0.100 (-0.148, -0.053)	0.000041*
Threonine	0.044 (0.021, 0.068)	0.000266*
3-hydroxybutyric acid	-0.150 (-0.237, -0.063)	0.000769
Asparagine	0.030 (0.012, 0.047)	0.000796
Guanidinoacetic acid	0.054 (0.021, 0.087)	0.001581
Citrulline	0.070 (0.026, 0.115)	0.001892
Free carnitine	0.033 (0.012, 0.054)	0.002175
Hydroxydecanoylcarnitine	-0.070 (-0.118, -0.023)	0.003709
Glutamine	0.023 (0.006, 0.039)	0.006757
Creatine	0.057 (0.016, 0.099)	0.006885
Uridine monophosphate	0.027 (0.007, 0.048)	0.01014
Glycine	0.033 (0.007, 0.058)	0.011068
3-hydroxyisobutyric acid	-0.049 (-0.086, -0.011)	0.011594
3-phenyllactic acid	0.061 (0.014, 0.109)	0.011765
CMPF[1]	-0.137 (-0.245,-0.03)	0.012445
2-hydroxy-3-methylbutiric acid	-0.058 (-0.105, -0.011)	0.015344
Hydroxydodecenoylcarnitine	-0.075 (-0.137, -0.013)	0.017916
Tyrosine	0.024 (0.004, 0.045)	0.020921
Butenoylcarnitine	-0.055 (-0.102, -0.008)	0.022623
Dodecenoylcarnitine	-0.059 (-0.11, -0.007)	0.025315
Ketoleucine	-0.03 (-0.057, -0.003)	0.02826
Alpha-ketoisovaleric acid	-0.03 (-0.056, -0.003)	0.028751
Asymmetric dimethylarginine	0.018 (0.002, 0.035)	0.030252
Uridine	-0.035 (-0.068, -0.003)	0.034513
Leucine	-0.023 (-0.046, -0.001	0.041282
Decenoylcarnitine	-0.046 (-0.09, -0.002)	0.041838
Ornithine	0.037 (0.001, 0.072)	0.04386
tetradecadienoylcarnitine	-0.053 (-0.105, 0)	0.049448
2-aminobutyric acid	-0.037 (-0.073, 0)	0.049939
Eicoseneoylcarnitine	-0.036 (-0.073, 0)	0.05045
Lactic acid	-0.037 (-0.075, 0)	0.050511
Trimethylamine-N-oxide	-0.083 (-0.166, 0.001)	0.05328
N –alpha acetyl-L-arginine	0.031 (-0.001, 0.062)	0.055828
Hydroxyproline	0.062 (-0.002, 0.125)	0.058796
Cholesterol sulfate	-0.04 (-0.081, 0.002)	0.060322
Pyroglutamic acid	0.021 (-0.002, 0.045)	0.070856
N-2-furoylglycine	0.121 (-0.014, 0.257)	0.079555
N-acetylglutamic acid	0.03 (-0.004, 0.065)	0.083373
Imidazole-4-acetic acid	0.036 (-0.005, 0.078)	0.088661
Cystine	-0.05 (-0.108, 0.008)	0.092408

Valine	-0.016 (-0.035, 0.003)	0.093601
Oleoylcarnitine	-0.028 (-0.062, 0.005)	0.095826
Hexadecadienoylcarnitine	-0.04 (-0.087, 0.008)	0.101163
N-acetylcitrulline	0.031 (-0.007, 0.069)	0.109125
Homocitrulline	-0.047 (-0.105, 0.011)	0.114967
Arginine	0.022 (-0.006, 0.051)	0.125281
Octanoylcarnitine	-0.044 (-0.099, 0.012)	0.126389
Hydroxytetradecenoylcarnitine	-0.033 (-0.076, 0.01)	0.128735
Octenoylcarnitine	-0.041 (-0.096, 0.013)	0.135082
Tetradecenoylcarnitine	-0.041 (-0.094, 0.013)	0.135491
N-acetylthreonine	0.015 (-0.005, 0.034)	0.139269
Tryptophan	0.016 (-0.005, 0.038)	0.141038
Creatinine	0.015 (-0.005, 0.035)	0.142657
Acetylcarnitine	-0.021 (-0.05, 0.007)	0.144418
Serine	0.014 (-0.005, 0.033)	0.153896
Palmitoleoylcarnitine	-0.03 (-0.071, 0.011)	0.15624
Nicotinamide	-0.042 (-0.102, 0.018)	0.170963
N-acetylmethionine	0.015 (-0.007, 0.036)	0.17853
Octadecanoylcarnitine	-0.022 (-0.053, 0.01)	0.18037
Linoleoylcarnitine	-0.022 (-0.057, 0.012)	0.207461
Xanthurenic acid	0.013 (-0.008, 0.034)	0.22025
Decanoylcarnitine	-0.033 (-0.087, 0.021)	0.22987
1-methyladenosine	-0.018 (-0.048, 0.011)	0.230374
Decatrienoylcarnitine	-0.051 (-0.135, 0.033)	0.235701
Arachidoylcarnitine	-0.019 (-0.05, 0.013)	0.24208
Urea	-0.012 (-0.033, 0.009)	0.249459
Succinic acid	-0.018 (-0.048, 0.013)	0.250773
N-alpha-acetyllysine	-0.019 (-0.051, 0.013)	0.251681
Palmityolcarnitine	-0.016 (-0.044, 0.012)	0.27488
3-aminoisobutyric acid	0.028 (-0.024, 0.08)	0.289111
Heptanoylcarnitine	-0.03 (-0.085, 0.025)	0.290565
Methionine sulfoxide	0.018 (-0.015, 0.051)	0.291983
Betaine	0.013 (-0.012, 0.038)	0.299764
Myristoylcarnitine	-0.02 (-0.059, 0.018)	0.300856
N-acetylputrescine	-0.015 (-0.043, 0.013)	0.305131
3-methyl-2-oxovaleric acid	-0.014 (-0.04, 0.012)	0.30538
1-methylnicotinamide	-0.035 (-0.102, 0.032)	0.306477
N,N-dimethylglycine	0.026 (-0.025, 0.077)	0.309646
Butyrylcarnitine	0.02 (-0.019, 0.059)	0.314701
4-pyridoxic acid	0.048 (-0.05, 0.146)	0.334901
Phenylalanine	0.009 (-0.01, 0.027)	0.355904
2-hydroxyisocaproic acid	-0.015 (-0.047, 0.017)	0.360686

C18:3 carnitine	-0.016 (-0.053, 0.02)	0.377092
Xanthine	0.024 (-0.03, 0.078)	0.380318
Mevalonic acid	0.033 (-0.04, 0.106)	0.382225
Kynurenic acid	-0.019 (-0.066, 0.029)	0.444633
4-oxoproline_pipecolic acid	-0.027 (-0.099, 0.044)	0.451506
N-epsilon-acetyllysine	0.007 (-0.012, 0.026)	0.461809
2,3-dihydroxybenzoic acid	0.04 (-0.075, 0.155)	0.491307
Dodecanoylcarnitine	-0.015 (-0.062, 0.032)	0.521192
Beta-alanine	-0.012 (-0.051, 0.027)	0.551536
Hydroxyoctenoylcarnitine	0.015 (-0.036, 0.066)	0.564465
Arachidonoylcarnitine	-0.012 (-0.052, 0.028)	0.566211
N-acetylaspartic acid	0.005 (-0.013, 0.024)	0.567771
Isoleucine	-0.007 (-0.03, 0.017)	0.574595
Malic acid	-0.012 (-0.056, 0.031)	0.579001
Glutaric acid	0.017 (-0.045, 0.078)	0.60072
Proline	0.007 (-0.019, 0.033)	0.602498
Phenylpyruvic acid	0.009 (-0.025, 0.043)	0.604217
Kynurenine	0.007 (-0.019, 0.033)	0.608161
Allantoin	0.007 (-0.021, 0.035)	0.610006
Decadienoylcarnitine	-0.014 (-0.067, 0.04)	0.619767
2,3-pyridinedicarboxylic acid	-0.012 (-0.06, 0.037)	0.642837
Taurine	0.01 (-0.033, 0.053)	0.661469
N-acetylserine	0.004 (-0.015, 0.022)	0.699988
Lysine	0.006 (-0.025, 0.037)	0.717826
Sarcosine	0.01 (-0.043, 0.063)	0.717886
S-adenosylhomocysteine	-0.007 (-0.048, 0.033)	0.729121
Glutamic acid	0.006 (-0.03, 0.042)	0.734843
N-acetyltryptophan	-0.005 (-0.04, 0.029)	0.758412
N-acetylalanine	0.002 (-0.013, 0.018)	0.772915
2-aminoadipic acid	-0.005 (-0.043, 0.032)	0.78199
Pantothenic acid	-0.005 (-0.045, 0.035)	0.805055
Citric acid	-0.004 (-0.033, 0.026)	0.816941
Uric acid	-0.003 (-0.027, 0.021)	0.819102
Isovalerylcarnitine	-0.006 (-0.061, 0.049)	0.826214
Hypoxanthine	0.005 (-0.049, 0.06)	0.84559
Trans aconitic acid	0.003 (-0.03, 0.036)	0.85435
C6:2 carnitine	0.009 (-0.099, 0.117)	0.865483
Riboflavin	0.003 (-0.037, 0.044)	0.873936
Propionylcarnitine	-0.003 (-0.038, 0.032)	0.879086
Aspartic acid	0.003 (-0.041, 0.047)	0.894736
Symmetric dimethylarginine	0.001 (-0.022, 0.025)	0.902373
Inosine	-0.007 (-0.132, 0.117)	0.906531

Alanine	0.001 (-0.02, 0.023)	0.922676
1-methylhistidine	-0.002 (-0.041, 0.038)	0.932877
S-methylcysteine sulfoxide	-0.003 (-0.069, 0.063)	0.934997
Isocitric acid	-0.002 (-0.06, 0.055)	0.936644
Alpha-ketoglutaric acid	0.002 (-0.047, 0.051)	0.943576
3-methylhistidine	0.005 (-0.128, 0.137)	0.945936
Histidine	0.001 (-0.024, 0.026)	0.951888
Guanosine	0.004 (-0.151, 0.16)	0.954978
Ethanolamine	0 (-0.024, 0.023)	0.969259
S-methylcysteine	-0.001 (-0.057, 0.054)	0.969628
N-acetylornithine	0 (-0.056, 0.057)	0.990867

The effect of dulaglutide on the 2-year difference of the natural logarithm (ln) levels of each metabolite calculated as ln (2-year/baseline) adjusted for ln (baseline biomarker level), age, sex, white identification, systolic blood pressure, previous cardiovascular disease, current smoking, body mass index, HbA1c, eGFR, LDL cholesterol, and albuminuria is shown. The biomarkers are sorted from the lowest to highest P values for the beta coefficient; only the subset with a P value <0.05 is shown). Negative values denote a greater fall in level from baseline to 2 years with dulaglutide versus placebo. *P values that are significant at a level of 0.05/135 = 0.00037. [1]CMPF - carboxy-4-methyl-5-propyl-2-furanpropanoic acid