Supplemental Material

Vascular Effects of Ultrafine Particles in Type 2 Diabetics

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Methods. Immunostaining Method for Platelets and Microparticles
(adapted from Li et al. (Li et al. 1999))

1. Citrated whole blood was diluted 1/10 with HEPES buffer (minus Ca, pH 7.4) within 10 minutes of collection.

2. A 50 μl aliquot of diluted blood was added to 30 μl of titered antibody combination and incubated for 20 minutes in the dark at room temperature.

3. Three ml of HEPES buffer was added to stop the reaction.

4. If samples were to be counted, 50 μl of AccuCount beads (Spherotech, Lake Forest, IL) were then added.

5. Tubes were gently mixed by inversion and immediately read on a FACScan (BD Biosciences, San Jose, CA) flow cytometer optimized for platelet reading.

6. One drop of Blank Size Calibration Bead (Spherotech, BCP 10-5) was added to the first sample to enable a boundary (1μ) to be set between microparticles and platelets at the lowest edge of the blank beads. A live gate was set on the platelet region and 30,000 events in this region were collected.

7. Platelets were gated based on the scatter region and GPIb (CD42b) positivity.

8. Circulating MP were characterized as follows: Platelet origin (PMP), $CD42b^{+}/31^{+}$; endothelial origin (EMP), $CD42b^{-}/31^{+}$ or $CD62E^{+}$ (Jimenez et al. 2003); MP expressing P-selectin ($CD62P^{+}$MP), tissue factor ($CD142^{+}$MP), or CD40 ligand ($CD40L^{+}$MP).

9. Flow analysis was performed using CellQuest® (BD Biosciences, San Jose, CA) and/or FloJo® (TreeStar, Ashland, OR) software.

10. Geometric mean intensities for surface marker expression were converted to molecules of equivalent phycoerythrin (MEPE) using standard curves generated with Rainbow Beads, (Spherotech, Lake Forest, IL) as per the manufacturer's directions.

Table 1: Antibodies Used[a]

Marker(Clone)	Name	Cell Type (Function)
Anti-CD31 PE (L133.1)	PECAM-1	wide expression (migration)
Anti-CD42b FITC (HIP1)	GPIbα	platelets (part of vWF receptor)
Anti-CD45 PerCP (2D1)	Leukocyte Common Antigen	only leukocytes
Anti-CD62E PE (68-5H11)	Endothelial-Selectin	activated endothelium (leukocyte adhesion)
Anti-CD62P PE (AK-4)	Platelet-Selectin	activated platelets and endothelium (adhesion)
Anti-CD142 PE (HTF-1)	Tissue Factor	monocytes, platelets, endothelium and smooth muscle layer (initiates the coagulation cascade)
Anti-CD154 PE (TRAP-1)	CD40 ligand (CD40L)	T cells (immune responses) platelets (activation, binding to monocytes and tissue factor release)

[a]All antibodies were obtained from BD-Biosciences(San Diego, CA).
Abbreviations: PE, phycoerythrin; FITC, fluorescein isothiocyanate; PerCP, peridinin chlorophyll protein

Table 2: Commercial ELISA Kits

R & D Systems, Minneapolis, MN

E-selectin (sCD62E)

L-selectin (sCD62L)

P-selectin (sCD62P)

Interleukin-6 (sIL-6)

Intercellular adhesion molecule-1 (sICAM-1)

Vascular adhesion molecule-1 (sVCAM-1)

Serum amyloid-A (SAA)

C-reactive protein (CRP)

Enzyme Research Laboratories, South Bend, IN

D-dimer

von Willebrand factor (vWF)

DakoCytomation, Carpenteria, CA

Factor VII, Factor IX

Soluble markers were all run on stored, frozen plasma. Heparinized plasma was used for E-, L-, and P-selectins, IL-6, sICAM-1, sVCAM-1, SAA and CRP. Citrated plasma was used for the coagulation factors, vWF, sCD40L and plasma tissue factor.

Table 3: Effects of Exposure on Indicators of Platelet Activation.

	Exposure	Time after exposure				
		Baseline	0.5 hr	3.5 hr	21 hr	45 hr
CD40L[a]	Air	40.1±1.3	42.1±1.6	39.1±1.3	39.9±1.4	39.8±1.2
	UFP*	39.8±1.3	39.6±1.5	43.9±1.3	41.6±1.1	39.3±1.9
CD40L[b]	Air	22450±487	3392±632	3780±753	3216±456	3311±611
	UFP	2847±414	3746±530	4191±584	3197±460	3066±463
CD40L[c]	Air	714±167	779±179	826±198	718±106	685±179
	UFP**	673±152	694±161	654±163	541±136	660±166
CD62P[a]	Air	16.6±.5	17.2±.6	16.0±.5	16.6±.6	17.0±.6
	UFP	16.7±.7	16.3±.6	17.3±.8	16.8±.6	16.6±.8
CD62P[b]	Air	883±93	862±92	905±123	948±147	79±85
	UFP**	914±112	1082±153	989±135	1011±153	897±113
CD62P[c]	Air	271±59	249±48	253±55	246±42	351±107
	UFP#	299±44	357±76	332±88	277±80	273±62
CD62P/45[d]	Air	112±13	100±13	124±23	106±18	99±18
	UFP##	115±24	123±19	145±25	120±21	146±27

[a] Mean ± SE molecules of equivalent phycoerythrin (MEPE).

[b] Mean ± SE counts of positive platelets/ul blood.

[c] Mean ± SE counts of positive microparticles/ul blood.

[c] Mean ± SE counts of positive leukocytes/ul blood.

[*] p = 0.015 UFP*time interaction by Model 1 analysis.

[**] p = 0.0002 UFP*gender effect by Model 2.

[#] p = 0.003 UFP effect, 0.05 UFP*gender interaction and 0.046 UFP*age interaction by Model 2.

[##] p = 0.037 UFP effect by Model 1; p = 0.034 UFP effect and 0.016 UFP*gender interaction by Model 2.

Table 4: Effects of Exposure on Tissue Factor (CD142)

	Exposure	Time after exposure				
		Baseline	0.5 hr	3.5 hr	21 hr	45 hr
Platelet[a] (MEPE)	Air	33.4±1.2	34.9±1.4	33.1±1.3	33.6±1.1	33.3±.9
	UFP	32.4±1.2	33.1±1.1	36.0±1.3	34.7±1.0	32.9±1.4
Platelet[b] (Count)	Air	1116±171	1242±195	1524±247	1293±157	1628±273
	UFP	1244±107	1378±191	1356±166	1387±174	1442±174
Monocyte[a] (MEPE)	Air	247±28	238±26	235±27	252±27	254±29
	UFP	247±23	248±22	258±27	250±23	246±21
Microparticle[b] (Count)	Air	453±101	322±70	365±87	414±108	281±67
	UFP	216±44	278±45	331±80	184±29	295±82
Protein[c] (pg/ml)	Air	27.42±3.85	28.94±4.02	26.60±3.74	26.25±3.86	25.80±3.35
	UFP	26.40±2.62	27.42±2.79	25.23±2.70	25.19±2.82	23.39±2.90

[a] Mean ± SE molecules of equivalent phycoerythrin (MEPE).

[b] Mean ± SE number of positive events/μl blood.

[c] Mean ± SE tissue factor plasma protein/ml plasma.

Table 5: Effects of Exposure on Microparticle Counts

	Exposure	Time after exposure				
		Baseline	0.5 hr	3.5 hr	21 hr	45 hr
Platelet MP	Air	2464±495[a]	2957±613	2836±681	2629±582	2767±467
($CD42b^+/31^+$)	UFP	2673±549	2792±614	2915±659	2964±560	2618±539
Endothelial MP	Air	1010±268	775±193	1501±558	757±167	1064±359
($CD42b^-/31^+$)	UFP	973±171	912±197	1022±190	1067±207	1031±328
Endothelial MP	Air	1487±336	1206±298	2113±639	1972±553	1575±454
($CD62E^+$)	UFP	1440±254	1321±342	1565±218	1162±250	1355±351

[a] All values are mean ± SE number of positive events/ul blood

Table 6: Effects of Exposure on Soluble Markers

Marker	Exposure	Time after exposure				
		Baseline	0.5 hr	3.5 hr	21 hr	45 hr
vWF	Air	130±6[a]	124±8	125±7	121±7	132±7
(%)	UFP[*]	125±6	138±8[**]	126±7	129±8	133±6
D-Dimer	Air	409±78	379±67	373±63	413±71	338±44
(ng/ml)	UFP	346±43	342±41	358±51	370±62	351±57
Factor VII	Air	117±11	124±8	111±9	121±8	111±9
(ng/ml)	UFP	130±11	126±11	118±13	116±11	119±10
Factor IX	Air	7.8±0.6	8.0±.8	8.3±0.8	7.0±0.6	7.8±0.5
(μg/ml)	UFP	8.1±0.6	8.6±0.7	8.2±0.5	8.6±0.7	8.3±0.7
IL-6	Air	2.4±0.3	2.3±0.3	2.5±0.3	1.9±0.3	2.2±0.4
(pg/ml)	UFP	2.4±0.3	2.5±0.4	3.0±0.4	2.2±0.3	2.35±0.4
CRP	Air	1669±433	1812±506	1636±448	1597±434	1496±396
(ng/ml)	UFP	1994±552	2018±544	1802±511	1917±531	1836±481
sCD40L	Air	488±97	485±99	509±103	475±97	522±102
(pg/ml)	UFP[**]	528±102	493±100	479±98	454±90	490±98

Abbreviations: von Willebrand Factor, vWF; interleukin-6, IL-6; C-reactive protein, CRP; soluble CD40ligand (sCD40L); plasma tissue factor protein, sCD142.

[a] All values are mean ± SE

[*] p = 0.047 UFP effect by Model 1 analysis

[**] p = 0.021 UFP*gender interaction by Model 2 analysis

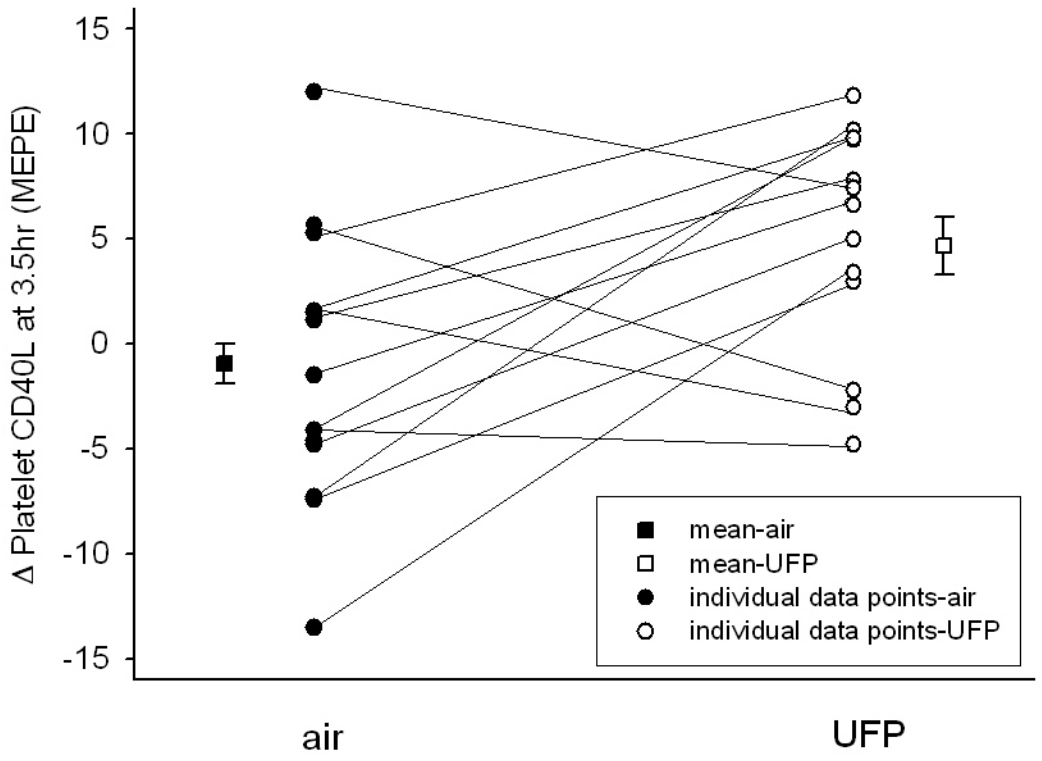

Figure 1: Individual changes in platelet expression of CD40L from baseline (pre-exposure) to 3.5 hours after exposure. Nine of the 13 subjects showed increased CD40L expression after UFP relative to air exposure.

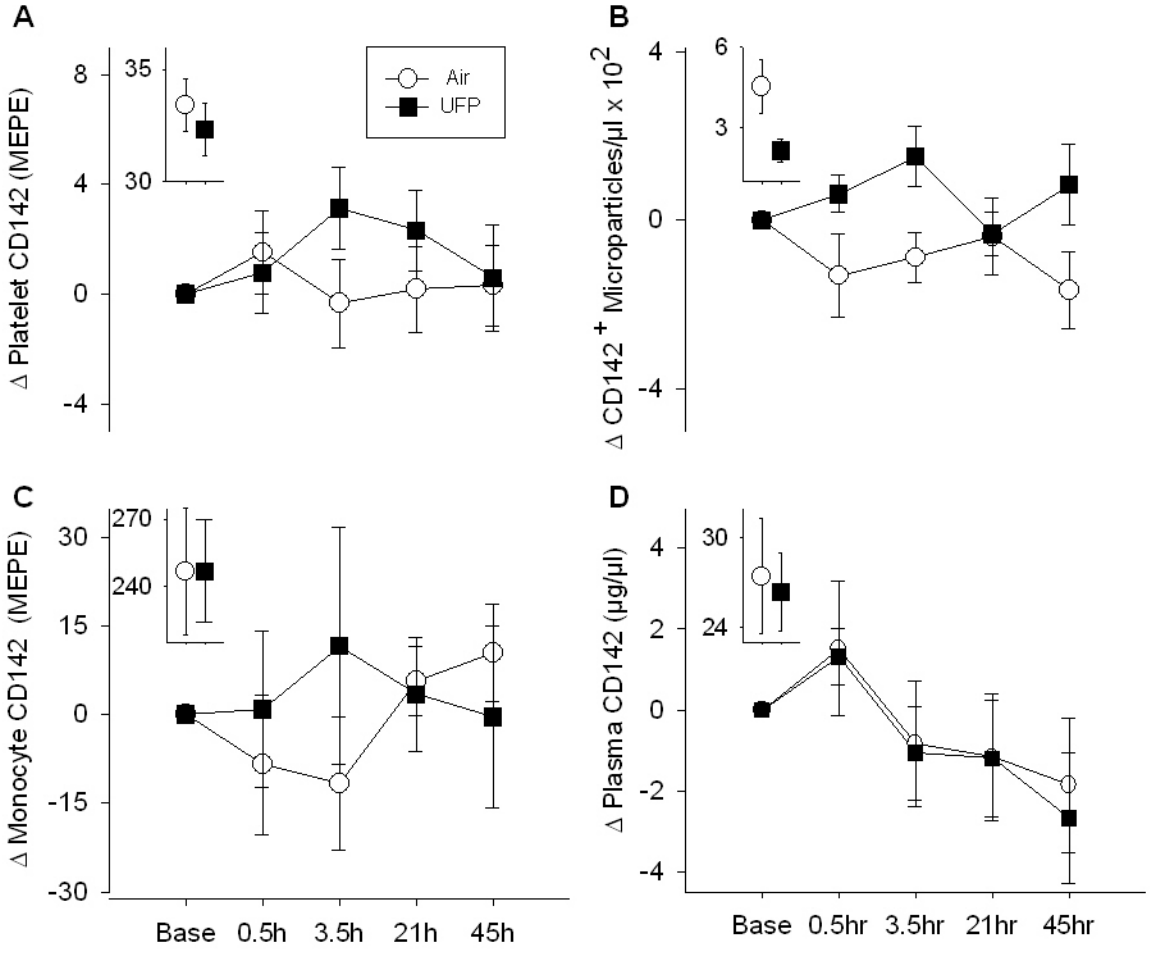

Figure 2: Changes in tissue factor (CD142) following exposure to UFP and air. A. Platelet surface expresion of tissue factor (n = 15). B. Number of circulating microparticles expressing tissue factor (n = 15). C. Circulating monocyte surface expression of tissue factor (n = 19). D. Plasma tissue factor (n = 19). Pre-exposure values are shown in the insets.

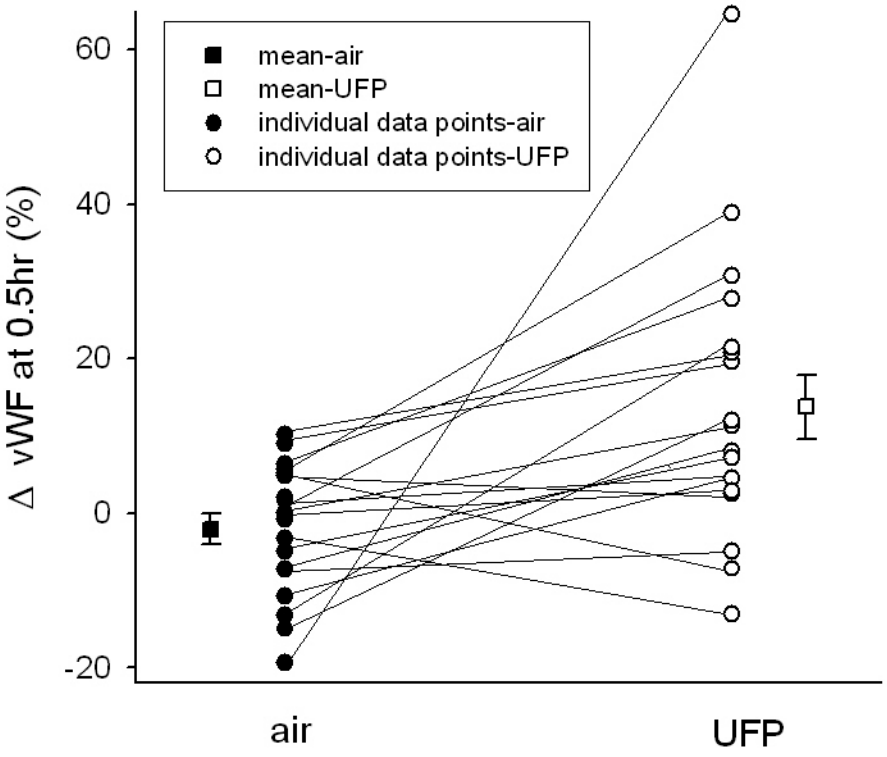

Figure 3: Individual changes in circulating vWF from baseline (pre-exposure) to 0.5 h after exposure. Fifteen of the 18 subjects increased vWF after UFP relative to air.

References

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