Supplementary Material to Schoergenhofer et al. “Prasugrel in critically ill patients” (https://doi.org/10.1160/TH17-03-0154)

Whole blood aggregometry

Whole blood aggregation was determined using the Multiple Electrode Aggregometry (MEA) on the Multiplate Analyzer (Dynabyte Medical). The system detects the electrical impedance change due to the adhesion and aggregation of platelets on two independent electrode-set surfaces in the test cuvette (22). A 1:2 dilution of whole blood anti-coagulated with heparin and 0.9% NaCl was stirred at 37°C for 3 min in the test cuvettes, ADP (adenosine diphosphate, 6.4µM) were added and the increase in electrical impedance was recorded continuously for 6 min (22). The mean values of the two independent determinations are expressed in units (U: tenth of area under the curve). The reference values for the test are as follows: ADP 29–118 U (23) (according to the manufacturer's information). A good reproducibility of MEA has been reported (<6% variability) (24).

Vasodilator-Stimulated-phosphor-Protein Phosphorylation (VASP-P) assay

The VASP-P enzyme-linked immune-assay (ELISA) was performed as reported previously (25). After activation with PGE_1 or PGE_1+ADP, incubation for 10 minutes (min) and lysis, samples were vortexed and stored at -80° Celsius. After thawing at room temperature (RT) samples were vortex-mixed. For antigen immobilization, 180µl of each sample were transferred to the plate and 180µl dilution buffer were pipetted into blank wells, which were covered and incubated for 30 min at RT. The wells were washed three times with each 300ml washing solution. For immobilization of immuno conjugate, 200 ml of diluted specified mouse monoclonal antihuman VASP-P ser 239 antibody coupled with peroxidase was added immediately. The wells were covered, incubated again for 30 min at RT and the washing step was repeated. Color development was performed by adding 200ml tetra-methyl-benzidine and incubating for 5 min at RT. The reaction was stopped with 100ml H2SO4 and a 2-min-incubation-step. Within 4h after stopping the reaction, the absorbance of the reaction product was

measured at 450nm. The PRI was calculated using optical density (OD) in the presence of PGE1 alone or PGE and ADP by means of the formula:

$PRI(\%)= [(OD450_{nmPGE1}-OD_{450nm(PGE1+ADP)}/(OD450_{nmPGE1}-OD_{450nmBlank})]*100$

Calculated values fell sometimes below zero in the ELISA. In this case the values were set to zero for all comparisons.

Platelet function Analyzer (PFA-100)

The PFA-100 (Dade Behring, Marburg, Germany) was used for measuring platelet function under high shear rates (5000-6000s^{-1}). Blood samples collected in 3,8% sodium citrate were used. The PFA-100 measures the time required for occlusion of the aperture by platelet plugs, which is defined as closure time (CT). The instrument aspirates a blood sample under constant vacuum from the sample reservoir through a capillary and microscopic aperture (147µm) cut into the membrane, which leads to high shear induced platelet plug formation (26). The membrane is coated with collagen/epinephrine (CEPI) or collagen/ADP (CADP) (27). The reference values for the CEPI-CT is 75-193 seconds (s) and 65-120s for CADP-CT (28). Individual day-to-day variability was reported for CEPI-CT (9%) (26). Published data have shown a satisfactory reproducibility of the test. Less than 2% of samples have shown a variation of more than 20% between the repeated measurements. When a cut-off of 105 seconds was applied PFA-100 had a 98% negative predictive value for the prediction of stent thrombosis and 88% negative predictive value for major adverse cardiovascular events (29).

Pharmacokinetics

Plasma concentrations of 3-methoxyphenacyl bromide (MPB)-prasugrel active metabolite were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) based on a published procedure (30). Blood samples (6 mL) were collected in pre-cooled EDTA tubes (BD Vacutainer, Becton Dickinson, Schwechat, Austria). To stabilize the free thiol group of the active prasugrel metabolite, 37.5 µL of 500 mM 3-methoxyphenacyl bromide (Sigma-Aldrich, Vienna, Austria) in acetonitrile (Sigma-Aldrich, Vienna, Austria) was added to each of the samples directly after collection. The blood samples were gently inverted

and immediately centrifuged at 1400 x g (15 minutes, 2-8°C) to separate the plasma. Aliquots were stored at -80°C and analyzed within 3 months.

The applied system consisted of a Symbiosis ALIAS chromatographic system (Spark Holland B.V., Emmen, Netherlands) and an AB Sciex detector (QTRAP 5500, AB Sciex, Framingham, MA, USA).

0.2 mL of an internal standard working solution (5 ng/mL of 13C6-prasugrel active metabolite, Alsachim, Illkirch Graffenstaden, France, stabilized with MPB, diluted with acetonitrile) was added to 0.1 mL of EDTA + MPB plasma. The precipitated samples were vortexed and centrifuged at 2400 x g. After a 1+1 dilution of the supernatant with water, 10 µL was injected into the LC-MS/MS using the electrospray ionization in positive mode. Chromatographic separation was achieved on a Kinetex Reversed Phase C18 column (particle size 2.6 µm, 50 mm x 3 mm, Phenomenex, Torrance, US) at a column temperature of 30°C using a mixture of 10 mM ammonium formate in water (phase A, gradient 0-60%) and ACN/MeOH (1:1, phase B, gradient 100-40%) at a flow rate of 0.4 mL/min. The transitions m/z 498.1 to m/z 248.1 for MPB-prasugrel active metabolite and m/z 504.1 to m/z 254.1 for MPB-13C6-prasugrel active metabolite were monitored in multiple reaction-monitoring mode.

A calibration curve (0.5, 1, 5, 10, 50, 100, 200 ng/mL) was constructed in plasma for prasugrel active metabolite (Alsachim, Illkirch Graffenstaden, France) stabilized with MPB.

Suppl. Table 1: Platelet reactivity and plasma concentrations of prasugrel active metabolite between patients treated with unfractionated heparin (UFH) or low-molecular weight heparin (LMWH).

Parameter	UFH (n=4)	LMWH (n=19)
PAM 0h [ng/mL]	0.2 (0.1-0.4)	0.6 (0.4-1.1)
PAM 2h [ng/mL]	7.5 (2.8-17.4)	5.7 (4.6-9.2)
PAM 24h [ng/mL]	0.4 (0.3-0.5)	0.7 (0.4-1.3)
MEA ADP 0h [AU]	71 (65-104)	52 (25-70)
MEA ADP 2h [AU]	67 (60-99)	47 (31-65)
MEA ADP 24h [AU]	70 (51-93)	55 (27-68)
PRI [%]	34 (21-54)	25 (16-32)
CADP-CT	70 (65-88)	161 (74-301)

Differences between groups were not significant. PAM= Prasugrel Active Metabolite, MEA ADP = ADP-induced whole blood aggregometry measured in the Multiplate Electrode Analyzer, PRI%= Platelet reactivity index in %, CADP-CT = platelet function under high shear rates measured by the platelet function analyzer 100 using ADP/collagen coated cartridges.

Suppl. Table 2: Platelet reactivity and plasma concentrations of prasugrel active metabolite between patients treated with high or low doses of norepinephrine, and patients withouth norepinephrine treatment.

Parameter	No norepinephrine (n=15)	Norepinephrine <0.15mcg/kg/min (n=4)	Norepinephrine >0.15 mcg/kg/min (n=4)
PAM 0h [ng/mL]	0.5 (0.3-0.6)	1.2 (0.55-2.2)	0.75 (0.35-1.8)
PAM 2h [ng/mL]	5.7 (3.8-9.8)	3.0 (0.7-5.4)	9.4 (6.4-11.5)
PAM 24h [ng/mL]	0.5 (0.3-0.7)	0.8 (0.6-1.4)	0.7 (0.4-1.3)
MEA ADP 0h [AU]	64 (45-70)	68 (41-85)	39 (23-64)
MEA ADP 2h [AU]	53 (42-66)	62 (37-75)	38 (23-69)
MEA ADP 24h [AU]	55 (47-68)	68 (41-85)	33 (17-68)
PRI [%]	25 (18-38)	24 (13-35)	26 (12-32)
CADP-CT	86 (68-233)	87 (69-200)	231 (151-301)
SOFA	5 (4-7)	10 (9.5-10.5)	10 (7.5-10.5)
SAPS 3	56 (44-66)	71 (68-75)	67 (60-71)
Platelet count [*10^9/L]	239 (119-289)	269 (228-283)	158 (151-292)

Differences between groups were not significant. PAM= Prasugrel Active Metabolite, MEA ADP = ADP-induced whole blood aggregometry measured in the Multiplate Electrode Analyzer, PRI%= Platelet reactivity index in %, CADP-CT = platelet function under high shear rates measured by the platelet function analyzer 100 using ADP/collagen coated cartridges, SOFA= Sequential Organ Failure Assessment Score, SAPS 3 = Simplified Acute Physiology Score 3.

Suppl. Table 3: Platelet reactivity and plasma concentrations of prasugrel active metabolite between patients treated continuously with opiates or without opiate treatment.

Parameter	No opiates (n=9)	Opiates (n=14)
PAM 0h [ng/mL]	0.5 (0.3-1.1)	0.6 (0.4-1.1)
PAM 2h [ng/mL]	9.9 (3.6-11.5)	5.2 (3.8-7.5)
PAM 24h [ng/mL]	0.5 (0.4-1.6)	0.4 (0.3-0.9)
MEA ADP 0h [AU]	65 (45-72)	61 (33-66)
MEA ADP 2h [AU]	57 (42-67)	53 (31-65)
MEA ADP 24h [AU]	55 (37-72)	62 (32-70)
PRI [%]	22 (16-32)	30 (20-37)
CADP-CT	120 (70-278)	104 (71-233)
Time after loading (days)	6 (4-16)	4 (3-10)
SOFA Score	6 (5-10)	6 (4-10)
SAPS 3 Score	60 (44-66)	65 (50-71)

Differences between groups were not significant. PAM= Prasugrel Active Metabolite, MEA ADP = ADP-induced whole blood aggregometry measured in the Multiplate Electrode Analyzer, PRI%= Platelet reactivity index in %, CADP-CT = platelet function under high shear rates measured by the platelet function analyzer 100 using ADP/collagen coated cartridges, SOFA = Sequential Organ Failure Assessment Score, SAPS 3 Score = Simplified Acute Physiology Score 3.