Supplementary Method 2

**Iontophoresis with laser Doppler flowmeter (LDF)**

Iontophoresis is used to deliver a locally applied drug for therapeutic and diagnostic purposes using electrically repulsive forces. Iontophoresis with charged vasoactive substances across the skin of the forearm can be achieved using a small electric current to achieve specific interference of skin microcirculatory responses, thereby avoiding systemic effects. Thus, iontophoresis has been suggested as a non-invasive and appropriate tool to determine endothelial dysfunction. The current strengths presently used for measurements well tolerated in the majority of the subjects, and only a mild pricking sensation during the measurement process has been reported in a few cases. The laser Doppler flowmeter (LDF) was used to measure blood flow in the small-sized blood vessels of the microvasculature close to the skin surface. Laser beams are produced by a semiconductor laser diode installed in the LDF probes, and these beams penetrate the skin and hit the red blood cells in the superficial vascularity after they are dispersed. The laser beams are then converted into scattered light by frequency variation (Doppler shift), which occurs in a linear proportion to the velocity of the red blood cells, allowing the change in light dispersion and intensity to be measured, which in this study was recorded as electric signals by a photo-detector. To measure endothelium-dependent and -independent response, iontophoresis with acetylcholine (Ach) and sodium nitroprusside (SNP) applied by LDF was performed, respectively. The combination of these techniques is a valid approach to measure of endothelial function.

All subjects sat in a comfortable chair and had a 20-min acclimatization period in a temperature-controlled room (22–24°C). After selecting an arm with no graft or fistula, the skin of the forearm was carefully cleaned using alcohol. A drug delivery chamber electrode (PF 383; Perimed, Järfälla, Sweden) linked with a different electrode (PF 384; Perimed) was attached to the volar surface of the forearm at a distance of 10-mm. One electrode for Ach was attached to the upper forearm and the other for SNP was applied at a lower site with a distance between the two of at least 10-cm. The laser Doppler probe connected to the LDF (Periflux PF4001, standard probe PF408, Perimed) was fixed within the drug chamber to explore the same small area of the skin. The laser Doppler outputs were recorded continuously with an interfaced computer equipped with acquisition software and recorded as arbitrary units (perfusion units, PU). Calibration was performed using a device composed of colloidal latex particles, the Brownian motion of which provided a standard value. The skin blood flow was determined as the mean value recorded during 60 sec or 180 sec at each delivery step. The absolute maximal response was defined as the flow that was reached after the last drug delivery. Before drug delivery, each drug delivery electrode chamber was filled with 62.5 μL of a 1% Ach and 1% SNP solution. A battery-powered iontophoresis controller (Perilout 328, Power Supply) was used to provide the current for drug administration. After registration of baseline flow, Ach was delivered using an anodal current; six doses (0.1 mA for 20 sec each) followed by another two doses (0.2 mA for 20 sec each) were delivered with a 60-sec interval between each dose. The 60-sec interval was needed to achieve a hyperemia plateau induced by Ach delivery. After a 1-min recovery period, SNP was delivered using a cathodal current; two doses (0.1 mA for 20 sec each) followed by one dose (0.2 mA for 20 sec) with a 180-sec interval between the two successive doses. The 180-sec interval was needed to achieve the steady state of the blood flow response following each SNP dose.

In order to eliminate baseline variability, the blood flow responses to locally delivered Ach and SNP were expressed as the ratio of changed PUs to the baseline PU. Similarly, the maximal flow increment following each of the eight iontophoretically applied Ach doses was defined as the endothelium-dependent response while the maximal response to SNP doses was defined as the endothelium-independent response.

**REFERENCES**