Original Communication

Bioavailability of naringenin chalcone in humans after ingestion of cherry tomatoes

Carina Kolot[1], Ana Rodriguez-Mateos[2,3], Rodrigo Feliciano[2], Katharina Bottermann[4], and Wilhelm Stahl[1]

[1] Institute of Biochemistry and Molecular Biology I, Medical Faculty, University of Düsseldorf
[2] Division of Cardiology, Pulmonology, and Vascular Medicine, Medical Faculty, University of Düsseldorf
[3] Department of Nutritional Sciences, School of Life Course Sciences, Faculty of Life Sciences and Medicine, King's College London, London, UK
[4] Institute for Cardiovascular Physiology, Medical Faculty, University of Düsseldorf

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Abstract: Chalcones are a type of flavonoids characterized by an α-β unsaturated structural element which may react with thiol groups to activate pathways such as the Nrf2-Keap-1 system. Naringenin chalcone is abundant in the diet but little is known about its bioavailability. In this work, the bioavailability of naringenin chalcone from tomatoes was investigated in a group of healthy men (n=10). After ingestion of 600 grams of tomatoes providing a single dose of 17.3 mg naringenin chalcone, 0.2 mg of naringenin, and 195 mg naringin plasma levels of free and conjugated naringenin and naringenin chalcone (glucuronide and sulfate) were analyzed by UHPLC-QTOF-MS at 0.5, 1, 3, and 6 h post-consumption. Plasma levels of conjugated naringenin increased to about 12 nmol/L with a maximum at about 3 h. Concentrations of free naringenin hardly elevated above baseline. Plasma levels of free and conjugated naringenin chalcone significantly increased. A maximum of the conjugated chalcone was reached at about 3 h after ingestion with an average concentration of about 0.5 nmol/L. No free chalcone was detectable at baseline but low amounts of the unconjugated compound could be detected with an average maximum of 0.8 nmol/L at about 1 h after ingestion. The data demonstrate that naringenin chalcone is bioavailable in humans from cherry tomatoes as a dietary source. However, availability is poor and intramolecular cyclisation as well as extended metabolism likely contribute to the inactivation of the reactive alpha-beta unsaturated reactive center as well as the excretion of the biologically active molecule, respectively.

Keywords: flavonoids, chalcones, naringenin, bioavailability, mass spectrometry

Introduction

Chalcones are secondary plant constituents and comprise a subgroup of the flavonoids. As a characteristic feature they exhibit an alpha-beta unsaturated structural element which is thought to be important for their biological activity e.g. activation of the Nrf2-Keap-1 system, likely involved in the cancer preventive properties of the compounds. Natural and synthetic chalcones are also studied as anticancer agents inhibiting cell proliferation, inducing apoptosis or modulating cell cycle progression with several signaling molecules including p53, Bcl-2 or TNF-related apoptosis-inducing ligand (TRAIL) as putative targets [1].

Among the dietary chalcones are cardamonin present in cardamom and other spices, xanthohumol found in hops and hops-derived products or naringenin chalcone present in some tomato varieties [2, 3]. They are intermediates in flavanone biosynthesis and cyclize spontaneously and/or enzymatically catalyzed to the respective three ring system. Thus, naringenin chalcone forms naringenin in an intramolecular reaction which is further conjugated to the glycoside naringin in the plant (Figure 1).

All of the three compounds may be present in fruit from specific tomato strains and in thereof produced dietary products [4]. In order to reveal their biological activities in-vivo, flavonoids must be absorbed while the functional groups remain unchanged. This has been discussed in context with the antioxidant properties of flavonoids and the contribution of free hydroxyl groups to radical scavenging [5]. Flavonoids are bioavailable from dietary products, absorbed in the gut and transported via the blood stream. The bioavailability of flavonoids varies with their structure and depends on a number of diet- (external) and host-related (internal) factors [6]. Most flavonoids exhibit a

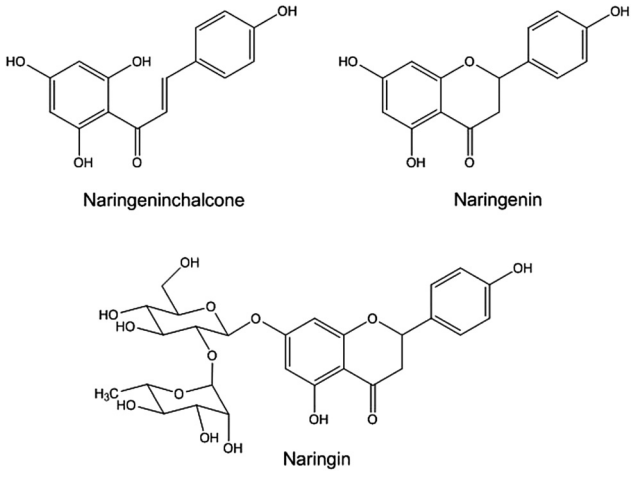

Figure 1. Structures of naringenin chalcone, naringenin, and naringin

pronounced first pass effect conjugating free hydroxyl groups to glucuronide or sulfate derivatives with only low amounts of the unconjugated parent compound remaining in the blood [7]. Although several studies on the bioavailability and biokinetics of three-ring flavonoids including naringenin have been performed, little is known on the uptake and metabolism of related chalcones following dietary intake in humans. Uptake and distribution of xanthohumol was determined after intake of hops-extract [8]. Bioavailability, metabolism and excretion of typical dihydrochalcones were evaluated following the consumption of rooibos tea [9]. For the first time, here we describe the bioavailability of naringenin chalcone from a dietary source: cherry tomatoes.

Materials and methods

Materials

Acetic acid was obtained from Carl Roth (Karlsruhe, Germany) and Oasis HLB μElution plates (2 mg sorbent per well, 30 μm) were from Waters (Eschborn, Germany). Milli-Q system (Merck KGaA, Darmstadt, Germany) ultrapure water was used. Naringenin chalcone was provided by Dr. Daniela Maydt. Naringin was from von Phytolab (Vestenbergsgreuth, Germany). Naringenin, beta-Glucuronidase/Sulfatase from Helix pomatia, Typ H-1 (partially purified powder) and all other chemicals and reagents were obtained from Sigma-Aldrich Co. (Taufkirchen, Germany).

Cherry tomatoes (about 5 kg) all of the same batch were obtained from a local distributer and analyzed for their content of flavonoids and carotenoids. For the intervention study a tomato puree was produced homogenizing equal amounts (w/w) of tomatoes and cold water in a blender for 5 min.

Methods

Study design

The study population comprised ten healthy, nonsmoking male adults (21-29 y) with a BMI of 20 to 28. Exclusion criteria were: intake of any medication or dietary supplements 4 weeks prior to the study. Participants were advised to avoid a flavonoid or carotenoid rich diet (especially tomatoes) one day before the start of the study. After overnight fasting, a blood sample was taken from each volunteer (t=0h). Than they ingested 600 g of the tomato puree within a period of maximum 10 min. Further blood samples (15 mL) were withdrawn at 0.5, 1, 3, and 6h after complete consumption of the tomato puree.

Blood was collected into 10 ml S-Monovette-tubes (Sarstedt, Nuembrecht, Germany) containing potassium EDTA as anticoagulant. Plasma was separated by centrifugation and stored at −80°C until analyses.

For analyses of flavonoids, plasma samples were divided into two portions. One portion was left untreated and the other was incubated with glucuronidase/sulfatase to remove conjugated glucuronic acid and sulfate. For further sample preparation for MS-analyses see below.

The study was approved by the Ethical Committee of the Heinrich-Heine-University Düsseldorf (Medical Faculty).

HPLC analyses of flavonoids, lycopene and α-tocopherol

An average sample of the cherry tomatoes (n=10) was collected from the entire tomato pool and used for analysis. The tomatoes were sliced in pieces and homogenized on ice with an Ultra Turrax. About 1 g of the homogenate was exactly weighed and extracted with 4 mL of methanol/water (70/30 v/v) in an ultrasonic bath. The mixture was centrifuged and filtered. The filtrate was directly used for HPLC analysis of flavonoids.

Separation was performed on a reversed phase column (LiChrosphere 100RP-18 5μm, 250x4 mm), protected by a guard column (4x4 mm) both from E. Merck (Darmstadt, Germany) with acetonitrile/water/acetic acid (37/60/3) as mobile phase, a flow rate of 1 mL/min and 20°C column temperature. HPLC system was a Merck-Hitachi L-7100 pump connected with a Merck-Hitachi diode array detector (Merck-Hitachi, Darmstadt, Germany). Injection volume was 50 μL and the detection wavelength for naringin and naringenin was set to 280 nm; for naringeninchalcone 342 nm. Concentrations of the analytes were calculated from external calibration curves.

Lycopene and α-tocopherol levels in tomatoes were analyzed according to [10]. Blood levels of lycopene and α-tocopherol were determined as described earlier [11].

UHPLC-Q-TOF-MS analyses of flavonoids in blood

Plasma was analyzed based on a previous validated μ-SPE coupled with UHPLC-Q-TOF-MS that allowed for high-throughput with slight modifications [12]. Briefly, plasma samples were thawed in ice and 600 μL were centrifuged at 15000 g for 15 min at 4°C. 350 μL of the supernatant were diluted (1:1) with 350 μL of phosphoric acid 4% and spiked with 167 nM of 2-hydroxy-4-methoxybenzoic acid as internal standard (IS). 600 μL were loaded on a 96 well μ-SPE HLB plate, washed with 200 μL of water and 200 μL of 0.2% acetic acid and finally eluted with 60 μL of methanol in each well. Extracted and concentrated plasma samples were analyzed with an Agilent 6550 iFunnel Accurate-Mass Quadrupole Time-of-Flight Mass Spectrometer (Q-TOF MS) after 5 μL injection, followed by separation on a 1290 Infinity UHPLC system (Agilent, Waldbronn, Germany) using a Zorbax Eclipse Plus RRHD column 2.1 x 50 mm, 1.8 μm (Agilent, Waldbronn, Germany). Column was at room temperature and the mobile phase consisted of 0.1% HCOOH (solvent A) and acetonitrile with 0.1% HCOOH (solvent B). The elution profile (flow rate of 0.2 mL/min) started at 39% solvent B and ran isocratically until 8 min. After 8 min, solvent B increased to 99% until 10 min with 2 min of equilibration time. The Q-TOF-MS parameters were as follows: negative mode, gas temperature 150°C, gas flow 11 L/min, nebulizer 25 psig, sheath gas temperature 350°C, sheath gas flow 12 L/min and Vcap 3000V. Mass accuracy was verified with a reference solution and the instrument was calibrated with a standard mixture to provide mass resolution >20,000 and mass accuracy <1 ppm. Phloretin prepared in post-extracted blank plasma (1420 nM) was used as a quality control (QC) for Q-TOF performance and stability evaluation. Ten concentration levels of naringenin and naringenin chalcone (0.1–500 nM) were prepared separately for each standard in mobile phase A: methanol (1:1). The identification of each compound was based on the extracted ion chromatogram (EIC) with a window of 2 ppm for each compound within a 1 min window of the RT of standards. The method was validated as previously described [1]. For the determination of LOQ the second most abundant isotope was used as qualifier (m/z = 272.0646), as well as the signal-to-noise ratio (SNR). The minimum concentration that elicited peaks with SNR >10 and in which the qualifier had a relative response within 80-120% of theoretical ion abundance was considered as the LOQ.

Data were analyzed and processed using Mass Hunter Workstation Quantitative and Qualitative Analysis software (version B.06.00, Agilent, Waldbronn, Germany).

HPLC-MS analyses comprised naringenin chalcone and naringenin assigned in the paper as free naringenin chalcone and free naringenin. The amount of naringenin chalcone and naringenin after treatment with glucuronidase/sulfatase is assigned as total naringenin chalcone and total naringenin.

Statistics

For plasma levels of all compounds, mean values and standard deviation (SD) were calculated. Each combination of two time points was compared using Wilcoxon's Signed Rank test. Differences were considered significant at $P < 0.05$.

Results

Ten adult, healthy male volunteers with an average age of 23.8 y and an average BMI of 23.0 participated in the study. Individual details are shown in Table 1. Also listed in Table 1 are the mean and individual baseline values of all compounds analyzed in the study. No unconjugated naringenin chalcone (free NC) was determined in any of the blood samples at baseline. Conjugated naringenin chalcone (glucuronides and sulfates) was present only in low amounts at an average of 0.1 nM (range 0 – 0.31 nM). Mean baseline levels of lycopene and α-tocopherol were determined as 0.7 and 19.7 μM, respectively.

A small batch of cherry tomatoes (about 5 kg) was obtained from a local supplier. Randomly picked fruits out of this batch were merged, homogenized and analyzed for their content of flavonoids, carotenoids and vitamin E (Table 2). The amount of free naringenin chalcone was 57.8 mg/kg fresh tomatoes whereas only low amounts of naringenin (0.6 mg/kg) were detected. Levels of the respective glycoside naringin were 649 mg/kg.

After an overnight fast, the volunteers consumed 300 g of homogenized cherry tomatoes mixed with 300 mL water within a period of 10 min. Thus, each volunteer consumed a single dose of 17.3 mg naringenin chalcone, 0.2 mg of naringenin, and 195 mg naringin with cherry tomatoes as a dietary source. Total intake of lycopene, β-carotene, and α-tocopherol was 12.4 mg, 1.9 mg and 2.0 mg, respectively.

Blood samples were analyzed for flavonoids by means of UHPLC-Q-TOF-MS. The coefficient of variation of the IS throughout the run was 17% and the QC accuracy was 104 ±3%. LOD and LOQ in plasma for the compounds were 0.08 and 0.3 nM respectively. The qualifier ratio at the LOQ level was within the acceptance criteria. Repeatability in plasma for a concentration of 1.5 nM was 5 and 10% for naringenin and naringenin chalcone, respectively. Linearity was obtained between LOQ and 500 nM for both

Table 1. Individual characteristics and individual baseline values of selected blood analytes determined in the study. Data are given as mean values ± SD.

No. of Subjects (gender)	Age (y)	BMI (kg/m²)	Naringenin chalcone (total) (nmol/L)	Naringenin (nmol/L)	Lycopene (μmol/L)	α-Tocopherol (μmol/L)
10 (male)	23.8 ± 3.0	23.0 ± 2.8	0.1 ± 0.1	0.8 ± 0.2	0.7 ± 0.3	19.7 ± 5.4

Table 2. Flavonoid, carotenoid and α-tocopherol content of cherry tomatoes used in the study Data are given as mean values ± SD (n=3).

Compound	Content (mg/kg wet weight)
Naringenin chalcone	57.8 ± 7.4
Naringenin	0.6 ± 0.2
Naringin	649 ± 24.3
Lycopene	41.4 ± 12.6
α-Tocopherol	6.7 ± 1.8

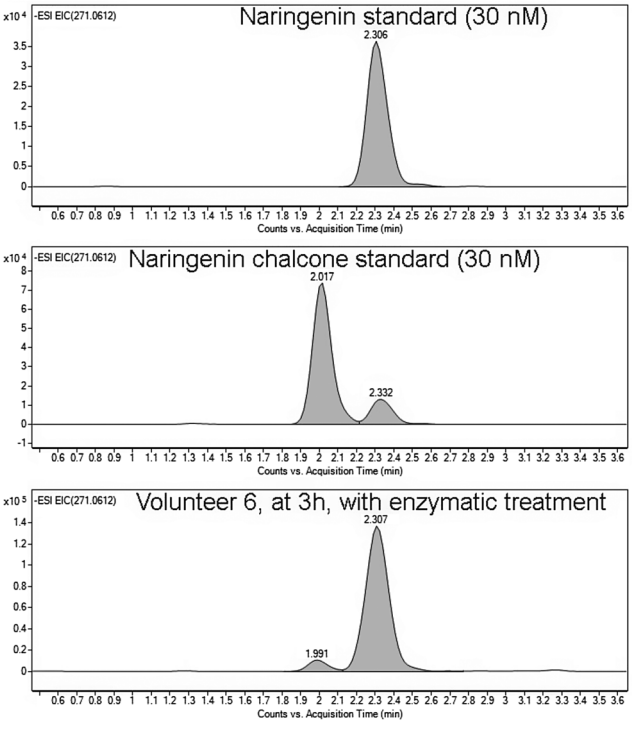

Figure 2. UHPLC-MS traces of naringenin and naringenin chalcone standards and a chromatogram of a plasma sample obtained from volunteer 6 (after treatment with glucuronidase/sulfatase)

compounds with $R^2 > 0.998$. Matrix effects in plasma were 31 and 22% for naringenin and naringenin chalcone, respectively. Accuracy of the method was evaluated by recovery tests and rates of 116±9% and 112±15% were achieved for naringenin and naringenin chalcone, respectively. For typical UHPLC-MS traces see Figure 2.

Upon ingestion of a single dose of 300 g cherry tomatoes, mean plasma levels of free and conjugated naringenin chalcone increased (Figure 3). Maximum plasma levels of the conjugated chalcone were reached at about 3h after ingestion with an average concentration of about 0.5 nmol/L. No free chalcone was detectable at baseline but low amounts of the unconjugated compound could be detected with an average maximum of 0.08 nmol/L at about 1h after ingestion.

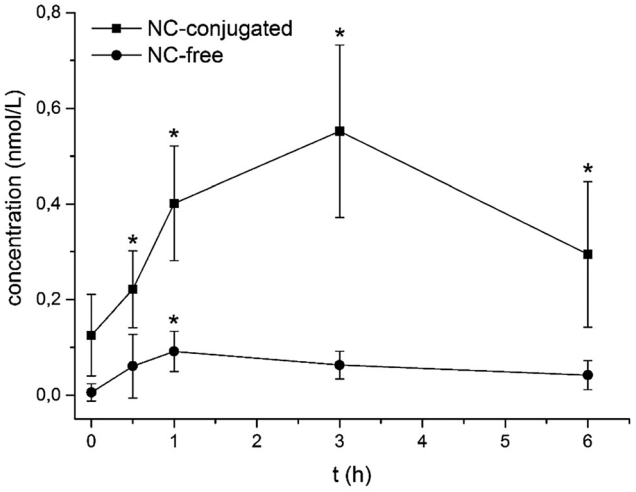

Figure 3. Plasma levels of free and conjugated naringenin chalcone (NC) after ingestion of 300 g cherry tomatoes. Data are given as mean values ± SD (n=10); *significantly different from baseline p<0.05.

Distinctively higher levels of conjugated naringenin were determined after consumption of the cherry tomatoes (Figure 4). After a continuous increase, a plateau of about 12 nmol/L was reached at about 3h. Concentrations of free naringenin were slightly elevated above baseline but much less pronounced compared to the corresponding conjugates.

The average plasma levels of lycopene and α-tocopherol did not significantly change after ingestion of the tomato puree and were comparable to the baseline levels given in Table 1 (data not shown).

In summary, the present data demonstrate that free naringenin chalcone as well as conjugated naringenin chalcone and naringenin circulate in human blood when the flavonoids are provided from a suitable dietary source.

Discussion

Cherry tomatoes proved to be a good dietary source of naringenin chalcone suitable for human intervention studies. With about 60 mg/kg the content exceeded the

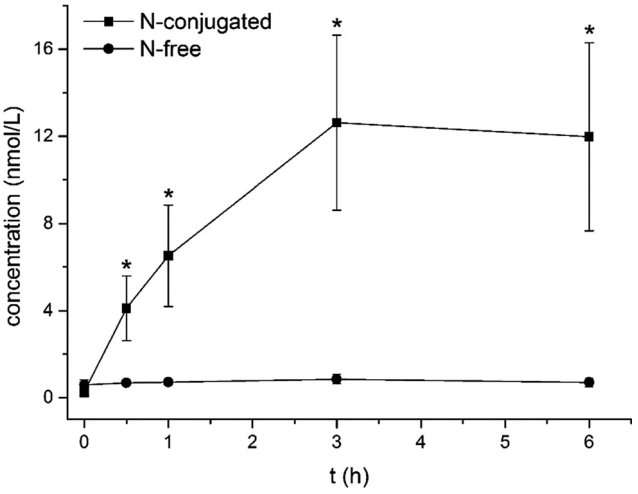

Figure 4. Plasma levels of free and conjugated naringenin (N) after ingestion of 300 g cherry tomatoes. Data are given as mean values ± SD (n=10); *significantly different from baseline p<0.05.

amount of naringenin. However, the level of glycosidic naringin was about 10-fold higher than that of naringenin chalcone. Depending on endogenous and exogenous factors, levels of flavonoids in tomatoes vary over a broad range. Concentrations between 9-182 mg naringenin chalcone and up to 13 mg naringenin per kg fresh weight have been reported [13]. Experiments with enzymatic hydrolysis suggest that about 70% of the total naringenin are present in the glycosylated form naringin [14] which is in accordance with the flavonoid pattern found in the cherry tomatoes used in the present study.

Upon ingestion of cherry tomatoes, total naringenin levels (free plus conjugated) in plasma increased which was almost exclusively due to elevated levels of conjugated naringenin (glucuronide and sulfate). Only minor amounts of free naringenin were detected. Flavonoid glycosides which are present in the diet are efficiently cleaved in food processing and during passage of the GI-tract [15]. With a determined first pass metabolism, the absorbed aglycons are subsequently conjugated to the respective phase II metabolites [16]. Thus, it is suggested that the majority of the total naringenin found in human plasma after ingestion of the tomatoes origins from naringin. The contribution of free naringenin from tomatoes to total naringenin is apparently negligible. However, cyclisation of naringenin chalcone from the dietary source to yield naringenin which is further conjugated in phase II likely contributes to total naringenin levels. Because all processes occur simultaneously during absorption and metabolism it is not possible to assign or quantify the contribution of each metabolic pathway.

In the present study, maximum levels of naringenin observed after ingestion of the dietary source are relatively low as compared to others. When naringenin was taken with a fruit juice mix (together with quercetin and hesperidin) at a single dose of about 12 mg, maximum blood levels of total naringenin levels were determined with about 220 nmol/L exceeding the levels here by the factor of 18 [17]. However, in agreement with our work, much lower levels (approximately 60 nmol/L) were measured when naringenin was provided with fresh cherry tomatoes at a single dose of about 20 mg [18]. It should be noted in this context that the bioavailability of tomato polyphenols depends on the composition of the food matrix, food processing and co-administration of additional dietary constituents such as lipids [19, 20].

Thus, it is difficult to compare absolute values from various studies.

After intake of the cherry tomatoes, we found elevated plasma levels of total naringenin chalcone (free plus conjugated). The major contribution was again from the conjugated forms. However, a significant increase was also determined for the free naringenin chalcone. Although the absolute maximum levels were only minor with about 0.08 nmol/L, this is the first time that an increase of free naringenine chalcone after application of a dietary source was demonstrated. Although little is known about the bioavailability of dietary chalcones, it was demonstrated that after ingestion of a hops extract the chalcone xanthohumol appeared in blood. Dose-dependently maximum blood levels were 12, 62 and 76 nmol/L for oral doses of 21.3, 42.6 and 85.2 mg xanthohumol, respectively [8]. Although dose levels were comparable, blood levels of naringenin chalcone were much lower than those of xanthohumol, which might be due to a pronounced intramolecular cyclisation of naringenin chalcone to naringenin.

Dietary chalcones have been associated with cancer prevention due to their biochemical activities. They are supposed to be antioxidants, inhibit inflammation, prevent proliferation but also modulate detoxification of carcinogens via the activation of phase II detoxifying enzymes [21]. The latter, if not all biological activities, are thought to be related to Michael type reactions of the alpha-beta-unsaturated structure of a chalcone with biologically relevant thiol groups. In context with secondary antioxidant effects and activation of phase II enzymes, apparently the activation of the Nrf2-Keap-1 system is of importance. Such biological effects have not yet been shown in humans, however, the present data demonstrate that the free chalcone of naringenin occurs circulating in the human organism after ingestion of the compound from a dietary source. Although the conjugated naringenin chalcones (glucuronides or sulfates) are prone to excretion, they still carry an intact alpha-beta unsaturated carbonyl system that might address biological targets in blood and tissues. Biological activities of polyphenolic glucuronides have already been described. Resveratrol glucuronides and

sulfates for example affects cell growth, status of the cell cycle and apoptosis in a human adenoma cell line [22].

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Author's contribution to the work: Concept and study design CK, WS; performance of the intervention study CK and KB; analyses of micronutrients RF, AR-M, CK; evaluation of the data CK, RF, AR-M, KB and WS; preparation of the manuscript CK, RF, AR-M, KB and WS.

Conflict of interest
The authors declare that there are no conflicts of interest.

Wilhelm Stahl
Institute of Biochemistry and Molecular Biology
University of Düsseldorf, Medical Faculty
POB 101007
D-40001 Düsseldorf
Germany
Tel. +49 211 811 2711

wilhelm.stahl@uni-duesseldorf.de

