Role of raloxifene on platelet metabolism and plasma lipids

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ABSTRACT

Background This study was performed to understand the metabolic effects of raloxifene, a selective oestrogen receptor modulator, on platelets in healthy non-obese postmenopausal women. The data were compared to untreated subjects.

Materials and methods Platelet nitric oxide activity (NO) and peroxynitrite level, platelet inducible and endothelial nitric oxide synthase expression and plasma lipids were evaluated at baseline and after 12 months of raloxifene or placebo treatment.

Results A significant increase of platelet NO and reduction of platelet peroxynitrite levels, as well as a decrease of inducible nitric oxide synthase expression, was observed 12 months after raloxifene therapy as compared to baseline or placebo treatment. Moreover, raloxifene treatment caused a significant increase in high-density lipoprotein cholesterol and a decrease of total cholesterol and low-density lipoprotein cholesterol were observed versus baseline values (P < 0.05). A significant positive correlation was observed between high-density lipoprotein cholesterol and platelet NO (r = 0.76, P < 0.005) in the raloxifene group.

Conclusion Our results showed that raloxifene improves platelet metabolism in healthy postmenopausal women through an increase of the bioavailability of platelet NO by a reduction of iNOS and the beneficial effects on lipid metabolism. This mechanism of action of raloxifene on platelet activity may explain some cardiovascular protective effects of this selective oestrogen receptor modulator.

Keywords Nitric oxide, NOS, peroxynitrite, platelets, postmenopausal women, raloxifene.


Introduction

Observational studies, as well as prospective randomized trials, reveal that the incidence of coronary artery disease is lower in premenopausal women than in age-matched men and postmenopausal women. These differences could be attributed to the oestrogens that seem to exert important physiological functions on endothelium-derived relaxing factor, smooth muscle proliferation, neurons and blood elements such as platelets and leukocytes [1].

Platelets play a vital role in vascular haemostasis. Their ability to aggregate and form a haemostatic plug must be carefully balanced with the necessity to maintain the fluid state of the blood and to avoid thrombosis. Platelet activation and recruitment are tightly regulated by products of the endothelium, including prostacyclin and nitric oxide (NO) [2].

NO is the only known endogenous formed radical acting as a signalling messenger. It is formed by nitric oxide synthases (NOS), which convert l-arginine to citrulline and NO. NO stimulates cyclic guanosine monophosphate (cGMP) production in human platelets leading to the activation of protein kinase C and the inhibition of platelet aggregation [3]. NO-induced inhibition of platelet aggregation involves a decrease of the intraplatelet Ca2+ concentration [4].

The most important NOS isoform within the cardiovascular system is the endothelial nitric oxide synthase (eNOS), that is regulated by Ca2+/calmodulin [5]. On the contrary, the inducible nitric oxide synthase (iNOS) is produced in response to cytokines. Under conditions of limiting l-arginine or favouring superoxide anion ‘bursts’, such as ischaemia-reperfusion, inflammation, hypercholesterolaemia, and angiotensin-induced hypertension, iNOS is expressed more than eNOS and it seems to produce superoxide (O2−) and NO at comparable rates [6]. This may have potentially deleterious consequences as O2− and NO react to form, quenched by intraplatelet glutathione, peroxynitrite (ONOO−), a very potent oxidant. Both NOS have been identified in human
platelets and megakaryocytoid cells [7]. In normal conditions, platelets do not express iNOS isoform, while they do express eNOS isoform that produces more NO than $\text{O}_2^-$. iNOS-dependent peroxynitrite production has been recently implicated in the pathophysiology of atherosclerosis, hypercholesterolaemia, hypertension and diabetes mellitus.

Several studies have shown that high plasma peroxynitrite concentration promotes platelet aggregation, while low peroxynitrite plasma concentration inhibits collagen-induced aggregation [8].

Hypercholesterolaemia induces an inhibition of endothelium-dependent vasorelaxation often referred to as endothelial dysfunction, which is one of the earliest manifestations of cardiovascular disease [9]. The pathogenesis of endothelial dysfunction involves a variety of oxygen-containing radicals and of non-radical small molecules with a more or less strong oxidative capacity [10] such as superoxide, hydrogen peroxide, hypochloride, NO and peroxynitrite. In platelets, hypercholesterolaemia induces a hyper-reactive state which significantly contributes to enhanced arterial thrombus formation through a reduced anti-aggregatory activity of prostacyclin, an increased serotonin release, and an enhanced response to platelet aggregators such as collagen and thromboxane [11].

Controversial results of hormone replacement therapy (HRT) have radically altered the contemporary understanding of its role in cardiovascular disease prevention [12]. Thus, raloxifene hydrochloride (RLX), a selective oestrogen receptor modulator (SERM), should be highly desirable for postmenopausal women [13] because of its oestrogen-agonistic effect on bone and lipid metabolism and of its oestrogen-antagonistic activity in the uterus and breasts. Moreover, SERM possesses vascular relaxing properties by an oestrogen receptor-dependent and NO-dependent mechanism [14].

The aim of the present study was to evaluate, for the first time, in non-obese postmenopausal women in respect to untreated subjects, the metabolic effects of raloxifene on platelets as they could be considered an ideal system to investigate the effects of drugs on platelet-derived NO. The following parameters were evaluated:

- The effects of RLX on platelet NO and peroxynitrite production;
- The changes in platelet NO and peroxynitrite synthesis with the activation of iNOS and eNOS;
- The correlation between platelet metabolism and plasma lipoproteins in healthy postmenopausal women.

**Subjects and methods**

**Patients**

Naturally healthy menopausal women between 56 and 64 years whose last menstrual period occurred at least 5 years before the beginning of the study and who had a T score bone mineral density $< -2$ DS at spine (L2–L4), without a history of fractures, were eligible to participate in the trial. Informed consent was obtained from all participants and the study was approved by the Local Ethics Committee. Patients were recruited from those reporting to the Centre for Osteoporosis and Menopause of the Unit of Endocrinology of the Department of Internal Medicine of Marche Politecnical University.

Exclusion criteria were: history of cardiovascular or cerebrovascular disease, deep-vein thrombosis or pulmonary embolism, bilateral oophorectomy, neoplasia, renal or hepatic dysfunction, malabsorption syndrome, smoking habits, abuse of alcohol or drugs, current use of cholesterol-lowering medication and HRT and cardiovascular medication. Moreover, patients were required not to have used any non-steroidal anti-inflammatory drugs or any other drugs for at least 6 months prior to, and during, study enrolment. This prospective, randomised, double-blind and placebo-controlled (CG) trial was performed on 80 healthy postmenopausal women. Postmenopausal status was defined by a follicle-stimulating hormone (FSH) level of more than 40 IU L$^{-1}$ and a serum oestradiol level of less than 91.8 pmol L$^{-1}$.

Forty postmenopausal women were treated with RLX 60 mg day$^{-1}$ for 1 year and 40 postmenopausal women were recruited as a control group (CG). Patients and controls were matched by age, body mass index (BMI) and postmenopausal duration (years). All subjects consumed an adequate daily calcium intake (above 1200 mg) and biochemical evaluation did not highlight hypovitaminosis D. Moreover, participants agreed not to alter their diet and exercise regimes during the study protocol. All patients completed the study.

Patients were asked to take one tablet or one capsule daily, at approximately the same time. To ensure compliance, patients were asked to return the packets containing placebos or medication at the end of every month.

**Biochemical evaluation**

After overnight fasting, before and after 12 months of treatment, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were measured, while low-density lipoprotein cholesterol (LDL-C) was calculated by the Friedewald formula [LDL-C = TC – HDL-C – (TG × 0.20)]. HDL-C was measured by photometric determination by a Trinder endpoint without prior separation (Direct HDL-Cholesterol, Bayer, Tarrytown, NY, USA).

Oestradiol and FSH (Automated Chemiluminescence Systems ACS Centaur; Bayer) were measured in all patients by chemiluminescent immunometric assays. The intra- and interassay coefficients of variation were below 5.2 and 7.3, respectively.
Platelet isolation
Peripheral venous blood was immediately mixed with anticoagulant citrate dextrose pH 6.5 (ACD). Platelets were isolated by differential centrifugation in anti-aggregation buffer (Tris-HCl 10 mm; NaCl 150 mm; EDTA 1 mm; glucose 5 mm; pH 7.4) according to Rao [15]. The anti-aggregation method involved a preliminary centrifugation step (200 × g for 10 min) to obtain platelet rich plasma (PRP). The platelets were then washed three times in anti-aggregation buffer and centrifuged in order to remove any residual erythrocytes. A final centrifugation at 2000 × g for 20 min was performed to isolate the platelets, which were immediately used for the experiments or stored at –80 °C for no longer than 15 days.

Determination of NOS activity
NO levels were measured in the supernates of lysed platelets, as previously described [16], using the Griess reaction [17]. Briefly, platelets were suspended in NO buffer (Hepes 25 mm, NaCl 140 mm, KCl 5.4 mm, CaCl2 1 mm, MgCl2 1 mm, pH 7.4) containing 1-4 mm NADPH and incubated for 1 h at 37 °C after the addition of L-arginine 100 μM. The reaction was then stopped by freeze-thawing the sample which was then sonicated. Each sample was incubated for 1 h at 37 °C after the addition of nitrate reductase (20 mU), which reduces nitrate to nitrite. After centrifugation at 1100 × g for 15 min, the supernatant was allowed to react with the Griess reagent (1% sulphanylamide/0.1% naphthylenediamine dihydrochloride/2.5% H3PO4). The chromophore absorption was read at 543 nm. Nitrite concentration was determined by sodium nitrite in water as standard. NOS activity was expressed in nmol NO produced · min⁻¹ · mg protein⁻¹. Protein concentration was determined as described by Bradford [18] using albumin as standard.

Preparation of 2,7-dichlorofluorescein-free base
2,7-dichlorofluorescein diacetate (DCFDA)-free base was prepared daily, by mixing 0.05 mL of 10 mmol L⁻¹ DCFDA with 2 mL of 0.01 N NaOH, at room temperature for 30 min. The mixture was neutralized with 18.0 mL of 25 mmol L⁻¹ phosphate-buffered saline (PBS) pH 7.4. This solution was maintained on ice in the dark until use [19].

Peroxynitrite production
Peroxynitrite production was evaluated by the use of a fluorescence probe DCFDA as previously described. Briefly, samples were incubated for 15 min with 5 μM DCFDA-free base at 37 °C. Then the DCFDA treated samples were incubated with or without the addition of L-arginine 100 mmol L⁻¹ and NG-monomethyl-L-arginine (L-NMMA) 100 mmol L⁻¹ for 15 min at 37 °C in the dark. After washing in PBS pH 7.4, the samples were sonicated. The mixture was then centrifuged at 2000 × g 5 min and the fluorescence was measured in the supernatant in a Perkin-Elmer LS-50B spectrofluorometer, at an excitation wavelength of 475 nm and emission wavelength of 520 nm.

Western blotting
Platelet rich plasma samples were lysed in RadioImmunoprecipitation Assay buffer (RIPA) lyses buffer containing 1 × PBS, 1/100 Igepal CA-630, 0.5/100 sodium deoxycholate, 0.1/100 sodium dodecyl sulphate (SDS), 10 mg mL⁻¹ Phenylmethylsulfonylfluorid (PMSF), aprotinin, 100 mmol L⁻¹ sodium orthovanadate and 4/100 protease inhibitor cocktails by microcentrifugation at 10 000 × g for 10 min at 4 °C. The supernatants were collected and treated with an equal volume of sample application buffer (125 mmol L⁻¹ Tris-HCl, pH 6.8, 2/100 SDS, 5/100 glycerol, 0.003/100 bromophenol blue, 1/100 β-mercaptoethanol). The mixture was boiled for 5 min; 15 μL of each sample was applied to each well of an 8/100 SDS polyacrylamide gel and electrophoresed for 1 h at 130 V along with a set of molecular weight markers (Broad Range, Sigma, St. Louis, MO, USA). The resolved protein bands were then transferred onto polyvinylidene fluoride (PVDF) membranes at 100 V for 60 min using a transfer buffer of 25 mmol L⁻¹ Tris base, 192 mmol L⁻³ glycin, and 20/100 methanol. The blots were blocked overnight at 4 °C with blocking buffer (5/100 non-fat milk in 10 mmol L⁻¹ Tris pH 7.5, 100 mmol L⁻¹ NaCl, 0.1/100 Tween 20). The blocking buffer was decanted and blots were incubated for 1 h at room temperature with primary antibody rabbit anti-endothelial nitric oxide synthase (eNOS, 1 : 1000, Chemicon, Temecula, CA, USA), rabbit anti-inducible NOS (iNOS, 1 : 1000, Chemicon) and rabbit antinitrotyrosine (N-Tyr 1 : 1000 Chemicon) diluted in blocking buffer. Positive controls were included in all experiments as provided by the manufacturer to confirm antibody specificity. As an internal control, blots were re-probed with an anti-β-actin antibody (Sigma). Blots were then washed using Tris Tween Buffer Saline (TTBS) washing buffer (10 mmol L⁻¹ Tris pH 7.5, 100 mmol L⁻¹ NaCl, 0.1/100 Tween 20) for 30 min with agitation and incubated with goat anti-rabbit secondary antibodies conjugated to hors eradish peroxidase (Sigma) diluted 1 : 5000 in blocking buffer for 1 h at room temperature followed by washes in TTBS. Peroxidase activity was revealed using 3,3′-diaminobenzidine (Sigma) as a substrate.

Densitometry was performed using software AMERSHAM Image Master 1D. All densitometric data are expressed as mean densities, defined as the sum of the gray values of all pixels in a selection divided by the number of pixels.
Statistical analysis

Statistical analysis was performed using the SAS statistical package (Statistical Analysis System Institute, Cary, NC, USA). Clinical characteristics of the women of both groups were compared by means of the unpaired Student’s t-test. Data were compared using paired and unpaired Student’s t-test. Correlations were performed by using Pearson’s coefficient. All values were reported as mean ± S.D. Significance was established at the level of P < 0.05.

Results

Clinical and biochemical characteristics

Tables 1 and 2 show clinical and biochemical data in the two randomized groups. At baseline there were no significant differences between the two randomized groups in relation to age, years from menopause, blood pressure, BMI, TC, HDL-C, LDL-C and TG. The level of FSH and oestradiol were also not significantly different between the two groups. BMI was the same before and after RLX therapy.

As described in the literature, platelet counts (279.3 ± 74.6 vs. 265.5 ± 57.2 10^9 L^-1, P > 0.05) were also not significantly different before and after RLX therapy [20].

NO activity and peroxynitrite level

At baseline the two groups had a similar platelet NO activity and peroxynitrite platelet level. After 12 months RLX-users showed a significant increase of platelet NOS activity compared baseline values (0.783 ± 0.040 vs. 0.348 ± 0.020 nmol · min^{-1} · mg protein^{-1}; P < 0.05) and a significant decrease of peroxynitrite platelet level (102.1 ± 34 vs. 159.6 ± 14 fluorescence arbitrary numbers; P < 0.05) compared with baseline values. Besides, at the end of the study, platelet NO level increased significantly in the RLX compared to controls (0.783 ± 0.040 vs. 0.380 ± 0.019 nmol nitrate · min^{-1} · mg protein^{-1}; P < 0.05), while platelet peroxynitrite levels decreased significantly in the RLX group as compared to the CG (102.1 ± 34 vs. 152.6 ± 17 fluorescence arbitrary numbers; P < 0.05) (Figs 1, 2).

Table 1 Baseline clinical characteristics on non-obese postmenopausal women treated either with raloxifene (RLX) or placebo (CG). Data are expressed as mean ± SD

<table>
<thead>
<tr>
<th>Variable</th>
<th>RLX</th>
<th>CG</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Women (n°)</td>
<td>40</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>56 ± 4</td>
<td>55 ± 3</td>
<td>ns</td>
</tr>
<tr>
<td>Postmenopausal duration (years)</td>
<td>7 ± 3</td>
<td>6 ± 2</td>
<td>ns</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Systolic</td>
<td>120 ± 5</td>
<td>125 ± 5</td>
<td>ns</td>
</tr>
<tr>
<td>Diastolic</td>
<td>80 ± 5</td>
<td>75 ± 5</td>
<td>ns</td>
</tr>
<tr>
<td>BMI (kg m^2)</td>
<td>24.9 ± 3.9</td>
<td>25.1 ± 2.2</td>
<td>ns</td>
</tr>
<tr>
<td>FSH (mIU mL^{-1})</td>
<td>68 ± 4</td>
<td>66 ± 5</td>
<td>ns</td>
</tr>
<tr>
<td>Oestradiol (pmol L^{-1})</td>
<td>83.4 ± 3.7</td>
<td>81.7 ± 3.4</td>
<td>ns</td>
</tr>
</tbody>
</table>

BMI, Body Mass Index (kg m^2); FSH, follicle-stimulating hormone.

Plasma lipids

After 12 months we observed a significant increase of HDL-C numbers; P < 0.05) compared with baseline values. Besides, at the end of the study, platelet NO level increased significantly in the RLX compared to controls (0.783 ± 0.040 vs. 0.380 ± 0.019 nmol nitrate · min^{-1} · mg protein^{-1}; P < 0.05), while platelet peroxynitrite levels decreased significantly in the RLX group as compared to the CG (102.1 ± 34 vs. 152.6 ± 17 fluorescence arbitrary numbers; P < 0.05) (Figs 1, 2).
as compared to baseline values (Table 2), while TG remained unchanged; a significant positive correlation between HDL-C and NO activity ($r = 0.76; P < 0.005; \text{Fig. 3}$); a significant negative correlation between NO activity and peroxynitrite ($r = -0.89; P < 0.0001 \text{Fig. 4}$) in RLX group.

**eNOS and iNOS expression**

Western blot analysis using anti-iNOS and eNOS monoclonal antibodies demonstrated that both isoforms were detectable in platelet lysates. In fact, in RLX-users the blot density of the bands corresponding to eNOS was unchanged after 12 months, while the blot density of the bands corresponding to iNOS was decreased after 12 months. No significant variation was highlighted in the CG (Fig. 5a,b).

**Discussion**

Cardiovascular diseases remain the largest cause of morbidity and mortality among postmenopausal women in Westernized societies. In myocardial infarction and unstable angina, as documented by angiographical and pathological studies, platelet aggregation and then the thrombus formation within a coronary vessel are the precipitating events [21].

Adhesion of platelets to the endothelium is prevented by endothelial cell production of prostacyclin and NO [2]. NO is an endogenous vasodilator and anti-aggregating substance produced in almost all human tissues.

Platelets are one potential target for oestrogen modulation within the cascade of factors involved in ischaemic atherosclerotic events. Oestrogens can modulate changes non-genomically (by affecting Ca$^{2+}$ flux, lipid metabolism, etc.) [22] and genomically seeing that oestrogen receptors have been cloned from megakaryocytes and platelets [23].

Nowadays, the evidence of the efficacy of oestrogens in the prevention of coronary artery disease has been called into question and, consequently, great efforts have been made to identify therapeutic alternatives such as several SERMs.
Figure 5 (a) Western immunoblots of inducible nitric oxide synthase (iNOS); protein expression in plasma samples obtained from raloxifene (RLX, n = 40); or placebo (CG, n = 40) treated subjects at baseline and after 12 months. β-actin was used as an internal control. (b) Western immunoblots of endothelial nitric oxide synthase (eNOS) protein expression in plasma samples obtained from raloxifene (RLX, n = 40) or placebo (CG, n = 40) treated subjects at baseline and after 12 months. β-actin was used as an internal control. (a,b) represent the densitometric analysis of iNOS and eNOS bands (P < 0.05)
that would have the benefit of oestrogens without their adverse effects [24].

Raloxifene (RLX), a benzothiophene derivative that binds with high affinity to the oestrogen receptor [25], is one of these SERMs that has been approved for the treatment of postmenopausal osteoporosis. In postmenopausal women this drug induces positive changes in the lipid profile and serum levels of fibrinogen and homocysteine without modifying the glucose metabolism and markers of inflammation [26–29]. Potential therapeutic benefits of this molecule in the prevention of cardiovascular disease have recently been evaluated in more than 7000 female osteoporotic participants in the Multiple Outcomes of Raloxifene Evaluation trial [30]. The authors, in raloxifene users of this post hoc analysis, did not find evidence of any early increased risk of cardiovascular events and highlighted a significant reduction in the risk of cardiovascular events in the subset of women with increased cardiovascular risk.

At present, the effects of RLX on cardiovascular risk are under evaluation in a large trial in postmenopausal women (Raloxifene Use in The Heart trial) with cardiovascular disease and breast cancer as primary endpoints [31].

To test this hypothesis several authors have studied the effects of RLX on NO release from cultured human umbilical vein endothelial cells and have shown a significant increase of NO production, due to an oestrogen receptor-dependent acute stimulation of eNOS enzymatic activity [32]. Others have compared the effects of raloxifene therapy and HRT on plasma NO products, endothelin-1 (ET-1) and prostacyclin plasma levels and endothelium-dependent vasodilation in postmenopausal women. These papers showed that both treatments influenced, comparatively, the endothelial function enhancing the ratio of NO to ET-1 [33,34], decreasing prostacyclin levels [33] and improving flow-mediated endothelium-dependent vasodilation [34]. In these trials the improvement of the endothelium-mediated vasodilation can be substantially referred to the reduction of ET-1, an endothelial constrictor factor, more than to an increase of endothelial NO.

The present study evaluates, for the first time, in non-obese postmenopausal women in relation to untreated subjects, the metabolic effects of RLX on platelets.

Our findings show that in healthy postmenopausal RLX users a significantly increased platelet NO activity, an unchanged eNOS expression, a reduced iNOS expression and a significant decrease in platelet peroxynitrite level can be achieved. These outcomes, taken together, strongly indicate that RLX-induced platelet NO production is due to the reduction of the iNOS isoform. This brings about a reduced iNOS-dependent superoxide formation, resulting in an underproduction of peroxynitrite that increases the bioavailability of platelet NO. In evidence, we have observed an inverse correlation between peroxynitrite and NO that suggests a greater NOS-dependent NO bioavailability that is not used to neutralize oxygen radicals.

Moreover, we have highlighted, in RLX-users, in accordance with several other authors, a decrease of TC and LDL-C, an increase of HDL-C levels and unaltered TG levels [28].

In addition our data shows, in the RLX group, a positive correlation between HDL-C and platelet NOS activity confirming that HDL-C decreases platelet function per se via an increase in nitric oxide synthase activity as observed by Chen and co-workers [35,36]. Since human platelets are unable to synthesize cholesterol, and platelet cholesterol content is regulated by plasma concentration of cholesterol, this positive correlation suggests that raloxifene may influence, indirectly, the platelet metabolism via an increased intracellular availability of L-arginine that reduces iNOS expression and platelet peroxynitrite levels. Furthermore, the inhibitory effect of HDL-C on platelet reactivity could also be due to the inhibition of LDL binding to its platelets’ receptor, the inhibition of oxidative modification of LDL or the removal of oxidized lipid components from oxidized LDL [37], thereby preventing these toxic lipids from activating the platelets.

In conclusion, our data highlight that RLX regulates platelet NO metabolism by a reduction of iNOS, and has beneficial effects on lipid metabolism. In fact, as platelet cholesterol content is regulated by plasma concentration of cholesterol [37], a reduction in plasma cholesterol levels may increase platelet NO production and contribute, thus, to negate the risk of cardiovascular events. These mechanisms raise the possibility that RLX may have the potential to be a cardioprotective agent with the benefit of no increased risk of cancer and other side-effects.

Moreover it is well known that RLX might have a neutral impact on cardiovascular disease, but our study is too short to demonstrate a realistic decrease in cardiac events. In fact, a recent study has shown that prothrombotic states may favour occlusive thrombi at sites occupied by atheromatous plaques. Platelet activation has received attention as an important determinant of arterial thrombogenesis but, although the evidence is still sparse, the globally available evidence suggests neutral or beneficial effects for SERMs [38].

Further work in humans is warranted to enhance our understanding of the mechanisms of the effects of RLX on platelet metabolism and activity to determine whether the effects of this drug may contribute to the reduction of cardiovascular related morbidity and mortality in postmenopausal women. Solid conclusions may be drawn only after the results of long term clinical studies, that are currently under way, are published.
References


