# Garlic and Cardiovascular Diseases

**S. Espirito Santo[1] • H-P. Keiss[2] • K. Meyer[3] • R. Buytenhek[1] •**

**Th. Roos[2] • V. Dirsch[2] • G. Buniatian[3] • C. Ende[3] • J. Günther[3] • K. Heise[3] • D. Kellert[3] •**

**K. Lerche[3] • S. Pavlica[3] • F. Struck[3] • E. Usbeck[3] • J. Voigt[3] • S. Zellmer[3] • J. M. G. Princen[1] •**

**A. M. Vollmar[2] • R. Gebhardt[3*]**

[1] TNO-Prevention and Health, Gaubius Laboratory, Zernikedreef 9, 2333 CK Leiden, The Netherlands
[2] University of Munich, Lehrstuhl für Pharamzeutische Biologie, Zentrum für Pharmaforschung, Butenandstrasse 5, 81375 Munich, Germany
[3] University of Leipzig, Institute of Biochemistry, Johannisallee 30, 04103 Leipzig, Germany

*Corresponding author*: * rgebhardt@medizin.uni-leipzig.de

## ABSTRACT

Based on extensive use in traditional medicine garlic has been claimed to prevent or mitigate cardiovascular disease. Lipid-lowering with particular emphasis on cholesterol and anti-inflammatory effects were considered as major mechanisms. This view, however, was challenged by an increasing number of clinical studies denying significant influence of various garlic preparations on these functions. Therefore, one of the aims of the European Garlic & Health project was the brought evaluation of the influence of well-defined garlic powders or of garlic-derived organosulphur compounds on surrogate markers of cardiovascular disease by *in vitro* assays, animal studies and a human intervention study. The results are briefly reviewed herein together with work published in parallel by other investigators. In conclusion, the investigations within the Garlic & Health project as well as the majority of independent studies provided compelling evidence against a beneficial influence of garlic powders or garlic constituents on risk factors and pathological aspects of cardiovascular disease in animals and humans.

## CONTENTS

## INTRODUCTION

The extensive use of garlic in traditional medicine strongly suggested a preventive effect of garlic on cardiovascular disease (CVD) (Agarwal 1996; Reuter *et al*. 1997). Clinical trials performed till the end of the last century, however, have led to conflicting results with respect to the influence of garlic consumption on plasma lipid and cholesterol levels (Orekhov and Grünwald 1997; Berthold *et al*. 1998; Isaacsohn *et al*. 1998; Superco and Krauss 2000), and conclusive evidenced-based data showing whether and especially how garlic constituents mediate cardioprotective effects were scarce. Available data suggest antithrombotic, lipid-lowering, antioxidant and immunomodulatory effects for garlic or selected garlic constituents (Agarwal 1996; Kyo *et al*. 2001; Hodge *et al*. 2002; Ariga and Seki 2006; Rahman and Lowe 2006). Recent developments in this field with particular emphasis on lipid-lowering, matrix-related and immunomodulatory effects that were one major focus of the European Garlic & Health project (Kik *et al*. 2001) will be reviewed below.

## LIPID-LOWERING EFFECTS

Until the mid-1990s, possible preventive effects of garlic regarding CVD were mainly attributed to its antithrombotic and lipid-lowering actions in animals and *in vitro* systems (Agarwal 1996; Reuter *et al*. 1997). Usually, *in vitro* studies using isolated and cultured hepatocytes or HepG2 cells revealed inhibitory effects of garlic extracts or garlic constituents at relatively high concentrations ranging between 0.1 and 4 mg/ml or 10 and 1000 µM (Gebhardt 1993; Gebhardt *et al*. 1994; Gebhardt and Beck 1996; Liu and Yeh 2000). Besides the fact that such concentrations are rather unlikely to be reached in blood after ingestion of garlic or garlic preparations, sporadic reports also suggested inhibitory ac-

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tions of garlic constituents on hepatocellular cholesterol biosynthesis (Sendl *et al.* 1992). Careful studies performed within the Garlic & Health project revealed the simultaneous presence of garlic constituents with both, enforcing and inhibiting properties, in one and the same extract prepared directly from garlic gloves. The balance of these constituents appeared to vary from one garlic variety to the other (**Fig. 1**) resulting in some kind of "activity fingerprint" specific for garlic accessions from different species and from differrent geographic locations (R Gebhardt, R Kamenetsky, HD Rabinowitch, pers. comm.). Nonetheless, when using primary cultured hepatocytes or HepG2 cells, the concentrations necessary for demonstrating enforcing or inhibitory effects were in the same high range mentioned above.

Further studies combining information about garlic plants with efficacy testing *in vitro* revealed a considerable impact of environmental factors on the qualitative and quantitative properties of several garlic varieties used commercially. Data from field trials on garlic production in France and Spain were compared to results from *in vitro* testing of the lipid-lowering potential of the respective garlic varieties. The garlic powders prepared according to standardized protocols were subjected to the comprehensive analysis of the spectrum of organosulphur compounds. Results indicated that planting time, climatic conditions, and sulphur fertilization of the soil differentially influenced the capacity of water-soluble extracts (prepared from varieties harvested under theses conditions) to modulate cholesterol biosynthesis (**Fig. 1**). Though there appeared to be a positive relation between the level of sulphur fertilization (up to 200 kg/ha) and the inhibition of cholesterol biosynthesis, this dependence was not observed when growth conditions considerably changed due to extreme weather conditions. These findings reflect the complex interdependence between plant metabolism and growth conditions. Despite reproducible results and application of superb analytics (Arnault *et al.* 2003, 2005) for characterising the garlic powders it was not possible, however, to attribute the observed effects to specific compounds or to the pattern of constituents. Obviously, the number of years for these observations was not sufficient to allow good correlations. Thus, the variable composition of garlic preparations has little predicttive value so far with respect to effectiveness. Solely, it seems certain that the sulphur content of the soil is a conclusive determinant of the quality of garlic and garlic preparations similarly as it was described with regard to the properties of onions (Randle *et al.* 1994; Hamilton *et al.* 1998). In consequence, repeated growing of garlic plants on the same field without additional fertilization may lead to a steady decline in garlic quality. This may be the case for many commercial garlic cultivations.
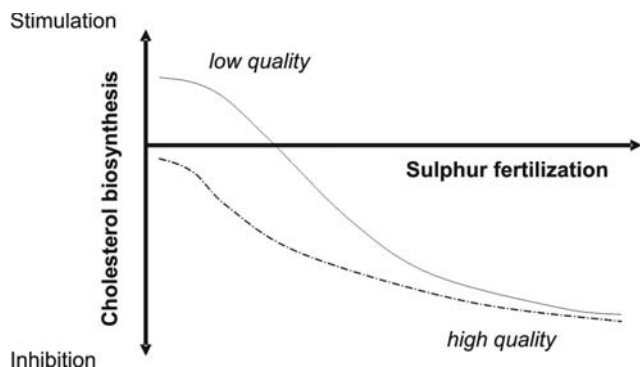


**Fig. 1 Schematic illustration of the dependence of garlic quality on soil sulphur content and fertilization.** Garlic plants grown in soil with low sulphur content show low quality, while those grown in soil with high sulphur content or fertilization show high quality. Different garlic varieties indicated by the solid and the dashed lines may depend differently on sulphur fertilization. Quality was evaluated *in vitro* by the effect of aqueous garlic extracts on hepatocellular cholesterol biosynthesis: garlic of low quality provokes stimulation, while garlic of high quality exerts inhibition.

**Table 1** Enhancement of AMP-dependent kinase (AMPK) activity by diallyl disulfide (DADS) and allyl mercaptan (AM) in rat hepatocytes cultured in the presence of different carbohydrates.

| Condition | Enhancement of AMPK activity | | | |
|---|---|---|---|---|
| | DADS | | AM | |
| | $EC_{50}$ value[a] ($\mu M$) | Enhancement (%) | $EC_{50}$ value[a] ($\mu M$) | Enhancement (%) |
| Control | $27.2 \pm 4.5$ | 37 | $42.9 \pm 8.8$ | 25 |
| Fructose | $1.7 \pm 0.6$** | 47 | $28.7 \pm 6.0$* | 33 |
| Galactose | $26.6 \pm 5.1$ | 31 | $43.8 \pm 8.3$ | 22 |
| Galactosamin | $4.2 \pm 1.8$* | 52 | $33.5 \pm 5.7$ | 29 |

[a] Values are means $\pm$ standard deviation of four independent determinations
* Different from controls, $p < 0.05$
** Different from controls, $p < 0.01$

A different line of evidence suggests a more subtle mode of action (Gebhardt 1997). Under specific conditions, diallyl disulphide (DADS) and perhaps few other garlic-derived organosulphur compounds at concentrations in the lower micromolar range may exert, at least *in vitro*, interesting modulating effects on hepatocellular cholesterol biosynthesis (Gebhardt 1995). Different experiments suggested that these effects occur indirectly by affecting the phosphorrylation status of HMGCoA reductase (Gebhardt 1995; Liu and Yeh 2002). The protein kinase involved was found to be AMP-dependent kinase (AMPK; Gebhardt unpublished results) the activity of which may be enhanced by DADS in the upper nanomolar range (**Table 1**). In the presence of fructose the dependence of this activation on DADS concentration is shifted to the left resulting in a considerably higher sensitivity. This is indicative of a dependence of this effect on the AMP content of the hepatocyte. Since AMPK is the fuel gauge of the cells (Hardie *et al.* 2006) and is activated only when the cell is in a low energy status, these mechanisms are rather unlikely to work in normal, healthy cells. However, it cannot be excluded that under conditions of increased cellular morbidity such as in diabetes or in other types of disease (associated with CVD) an increased sensitivity to certain garlic-derived organosulphur compounds may occur. Indications for such an increased susceptibility have been reported in streptozotocin-induced diabetic rats (Patumraj *et al.* 2000).

As obvious from the studies discussed above, *in vitro* experiments seem of limited value with respect to the question of whether garlic preparations may exert beneficial effects on CVD. Therefore, animal studies were conducted in the framework of the Garlic & Health project, in order to investigate lipid-lowering effects of garlic under *in vivo* conditions (Espirito Santo *et al.* 2004a). For this purpose, APOE*3-Leiden mice were used, a mouse model of mild hyperlipidemia that allows the titration of plasma lipids to selected levels relevant to conditions in humans by the addition of cholesterol and fat to the diet (Van Vlijmen *et al.* 1994). These mice were fed a garlic-derived sulphur-rich compound, either allicin (0.29 g/l drinking water) or DADS (0.27 g/kg diet) or powdered garlic, of either the commercial product Kwai or the garlic variety Morado de Cuenca (42 g/kg diet each; Espirito Santo *et al.* 2004a). It is important to note that all mice had access to a daily comparable dose of 44 mg/g allicin equivalent and that the European garlic variety 'Morado de Cuenca' was selected because it was fertilized with higher levels of sulphur during cultivation. When fed a nonpurified diet for 4 weeks, followed by a Western diet for 8 weeks, both supplemented with the garlic-derived materials, no consistent effects on plasma lipids could be observed (Espirito Santo *et al.* 2004a). There were also no changes in lipoprotein profiles which are markers for whole-body cholesterol synthesis and intestinal sterol absorption. These results strongly indicate that the postulated effects of garlic on CVD are not caused via modulation of plasma lipid levels. Similar results on the effects of allicin at a 5-times lower dose on lipid profile were reported by Abramovitz *et al.* (1999).

One of the major advantages of this animal study (Espi-

rito Santo *et al.* 2004a) was the use of specified garlic-derived sulphur-rich compounds and chemically well-characterized garlic powders of high quality. Unfortunately, similar detailed information on garlic composition is available only for a few (Abramovitz *et al.* 1999; Ali *et al.* 2000) but not for many other animal studies showing opposite results (Augusti *et al.* 2001; Slowing *et al.* 2001; Durak *et al.* 2002; Jabbari *et al.* 2005) with the consequence that the latter are less conclusive and cannot provide a basis for predictions of garlic efficacy in general.

## MATRIX-RELATED EFFECTS

Disturbance and reconstruction of the extracellular matrix (ECM) of the vessel wall is a common phenomenon in CVD involved in plaque formation and rupture. Accordingly, matrix metalloproteinases (MMPs) and particularly their inhibitors have been discussed with respect to atherosclerosis and myocardial infarction (George 2000; Creemers *et al.* 2001; Lijnen 2001). We have explored the possibility that garlic-derived compounds may influence biosynthesis of MMPs and of their natural inhibitors, the TIMPs (Meyer *et al.* 2004). Using human umbilical endothelial cells (HUVEC cells) it could be demonstrated by ELISA and activity assays that DADS at non-toxic concentrations starting at 10 μM selectively suppresses secretion of MMP-2 and TIMP-1. In cultures induced with forskolin, phorbol 12-myristate 13-acetate, and tumour necrosis factor-α, DADS induced reduction of MMP-9 and TIMP-1 in a concentration-dependent fashion. No effect of DADS was seen on MMP-1 and 13. Allyl mercaptan (AM) and S-allylcysteine (SAC) were ineffective in this assay (Meyer *et al.* 2004). These results leave it open of how the balance between MMPs and their inhibitor is changed, particularly in the context of an artherosclerotic tissue, because secretion of different MMPs is affected to a different degree. Besides interference with MMP synthesis and secretion, other possible mechanisms for the interference with the ECM have been reported. For instance, experimental nanoplaque formation by adsorption of proteoheparan sulphate to hydrophobic silica which is supported by a LDL plasma fraction from healthy probationers was inhibited by an aqueous garlic extract preferably at 1 mg/ml similar to a HDL fraction (Siegel *et al.* 2004).

## ANTI-ATHEROSCLEROTIC EFFECTS

Besides high LDL-cholesterol levels, certain other risk factors have been linked to CVD. Amongst others, hyperhomocysteinemia was found to be an independent risk factor for CVD. In an animal study using folate-deficient diets to induce hyperhomocysteinemia, supplementation of the diets with aged garlic extract (AGE) at 4% (w/w) was found to decrease homocysteine levels in plasma by 30% (Yeh and Yeh 2006). In an *in vitro* study, Ide *et al.* (2006) showed that expression of CD36 by human monocyte/macrophages (THP-1) was increased by high levels of homocysteine (200 μM) and significantly suppressed by coincubation with AGE (5 g/l). Though both studies suggest the possible contribution of AGE to the reduction of development and consequences of hyperhomocysteinemia, it has to be kept in mind that the doses of AGE applied are extremely high and probably without relevance for the human situation.

With respect to isolated garlic derived compounds, studies in mice on the effect of allicin on the formation of fatty streaks were published (Abramovitz *et al.* 1999). Although the lipid profile did not change, a significant reduction of the area of fatty streaks was found. Within the Garlic & Health consortium another mouse study was performed on this subject (Espirito Santo *et al.* 2004b). Using APOE*3-Leiden mice the possible atheroprotective effects of the garlic powder Printanor at two different doses and the AGE "Kyolic" were tested. No influence of either material on the atherosclerotic lesion type, area or composi-

tion was detected after 28 weeks of treatment (Espirito Santo *et al.* 2004b). In addition, other relevant parameters like plasma lipids, markers of inflammation, and vascular activation did not change, while an increased fecal fatty acid excretion was obvious. In conclusion, there is consistency in the absence of any influence of garlic material on lipid profiles, whereas conflicting results exist with respect to an antiatherosclerotic function exerted possibly via mechanisms other than lipid-lowering.

## IMMUNMODULATORY EFFECTS

With the discovery of redox-regulated transcription factors, such as NF-κB or AP-1, and their central role in inflammatory disorders, including CVD (de Martin *et al.* 2000; Li and Verma 2002; de Winther *et al.* 2005; Liu *et al.* 2005), garlic and garlic constituents were discovered as potential antioxidants and *in vitro* anti-inflammatory compounds. In 1998, allicin and ajoene were reported to inhibit lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) expression in murine macrophages (Dirsch *et al.* 1998). Effective concentrations had been found in the lower μM-range, with ajoene ($IC_{50}$ 2.5-5 μM) being more effective than allicin ($IC_{50}$ 15-20 μM). The exact mechanism how this effect occurs is, however, yet unknown. Since iNOS expression is largely under the control of NF-κB in LPS-activated RAW264.7 macrophages (Xie *et al.* 1994) an inhibitory effect of allicin or ajoene on NF-κB can not be excluded from this study. In addition, ajoene was shown to dose-dependently reduce prostaglandin $E_2$ release from LPS-activated RAW264.7 macrophages with an $IC_{50}$ value of 2.4 μM (Dirsch and Vollmar 2001). SAC, which is present especially in AGE is a significant antioxidant and reported to inhibit the redox-sensitive transcription factor NF-κB (Geng *et al.* 1997; Ide *et al.* 2001; Borek 2006). In human Jurkat T cells activated with TNF-α or $H_2O_2$, SAC (1-2 mg/ml; 2 mg/ml refers to 12.4 mM) dose-dependently reduced NF-κB binding activity as detected by electrophoretic mobility shift assay (EMSA) (Geng *et al.* 1997). Next to the fairly high concentrations used in this study, it may be worth to mention that SAC was preincubated for 24 hours before activation of Jurkat T cells. The same group showed some years later that SAC (2.5-20 mM) reduced NF-κB expression in TNF-activated HUVEC (Ide *et al.* 2001). The detected NF-κB subunit, however, was not specified. Using also TNF-α activated HUVECs, Keiss (2003) was unable to detect an inhibitory effect of SAC (0.1-10 mM) on the NF-κB binding activity by EMSA although the used positive control (parthenolide 0.1-10 μM) was effective. Next to SAC, further garlic constituents existing either genuine, such as γ-glutamylcysteine, or compounds that have been described as metabolites after garlic consumption, such as DADS, AM, or allyl-methyl-sulfone ($AMSO_2$) (Koch 1996; Germain *et al.* 2002) were examined for a potential NF-κB inhibitory activity. None of the examined compounds, DADS (5-20 μM), AM (5-20 μM), γ-glutamylcysteine (1 nM-1 mM; a major constituent of whole and crushed garlic gloves) showed a significant inhibitory effect on TNF-α-induced NF-κB binding activity (EMSA) in HUVEC; neither did an aqueous garlic extract (0.01-1 mg/ml) (Keiss 2003; Dirsch *et al.* 2004). Putative effects of garlic metabolites on the transactivation activity of NF-κB were examined by a NF-κB-driven luciferase reporter gene assay in human embryonic kidney cells (HEK293). Again, there was no significant inhibitory activity detectable for the metabolites, DADS (1-100 μM), AM (1-100 μM), $AMSO_2$ and the garlic compound allicin (Dirsch *et al.* 2004; Keiss 2003). Moreover, neither aqueous (1-1000 μg/ml) nor DMSO (1-500 μg/ml) extracts from several garlic powders prepared within the Garlic & Health consortium showed any effect on TNF-α-induced NF-κB transactivation activity (Keiss 2003). The endothelial adhesion molecules E-selectin and ICAM-1, both involved in pro-inflammatory processes (Hope and Meredith 2003) are largely NF-κB regulated. Thus, in the same study the influence

of DADS and AM, on the expression level of these adhesion molecules was examined by flow cytometry after activating cells for 4 hours (E-selectin) or 16 hours (ICAM-1) with TNF-α. At a concentration of 5-20 μM neither DADS nor AM inhibited E-selectin or ICAM-1 expression in HUVEC (Keiss 2003; Dirsch *et al.* 2004). Extending the preincubation period of garlic compounds to 24 hours and increasing the concentration up to 100 μM of DADS or AM did also not lead to a reduction in TNF-α-induced E-selectin or ICAM-1 expression in HUVEC. A study by Campbell *et al.* (2001) showed that also AGE ("Kyolic" 0.1-5%) had no effect on the expression of ICAM-1 in cultured endothelial cells. In accordance with that, Kyolic showed also no effect on the adhesion of leucocytes on the endothelium detected by scanning electron microscopy in this study. Interestingly, this group found that 1% "Kyolic" maintains the contractile smooth muscle cell (SMC) phenotype of SMC isolated from rabbit aorta and completely blocked DNA synthesis in these cells (Campell *et al.* 2001). A very recent publication again addressed the question as to whether garlic may inhibit endothelial adhesion molecules (Rassoul *et al.* 2006). This group used interleukin (IL)-1α-activated human coronary artery endothelial cells and employed an aqueous garlic powder extract (0.25-4.0 mg/ml) for 4 days before cells were activated with IL-1α. Under these conditions they found an inhibitory effect that was most pronounced at 4 mg/ml water-soluble garlic extract. These examples show that *in vitro* effects described for garlic extracts or constituents on NF-κB activity or the expression of NF-κB-regulated genes are highly dependent on the *in vitro* conditions used (cell system, inflammatory stimulus, and preincubation time of test compounds, among others).

Next to adhesion molecules pro- or anti-inflammatory cytokines are considered promising targets for a putative anti-inflammatory effect of garlic. Hodge *et al.* (2002) treated human whole blood and peripheral blood mononuclear cells with crushed garlic extract (≥ 0.1 μg/ml; ≥ 10 μg/ml) and measured pro- as well as anti-inflammatory cytokine production. They found an inhibition of Th1 and pro-inflammatory cytokine production while anti-inflammatory IL-10 was upregulated, although, described effects occurred at quite divers concentrations. A similar model was used by Keiss *et al.* (2003) to detect a putative indirect effect of garlic extracts or constituents on NF-κB activity. Human blood samples from healthy volunteers were incubated in the absence or presence of LPS with garlic powder extracts (DMSO; 10 or 100 μg/ml) or garlic metabolites (DADS, AMSO₂, allicin, γ-glutamylcysteine) for 20 hours. Thereafter, pro- (TNF-α, IL-1β) and anti- (IL-10) inflammatory cytokines were quantified in cell-free supernatants by ELISA. In a second step, these blood supernatants were added to HEK293 cells transfected with a NF-κB-driven luciferase reporter gene and NF-κB transactivation activity was measured. Garlic powder extracts (10 and 100 μg/ml) indeed significantly reduced LPS-induced TNF-α and IL-1β levels in human whole blood (**Fig. 2A, 2B**). This effect could be at least partly attributed to garlic organosulfur compounds since extracts from sulphur fertilized garlic reduced pro-inflammatory cytokine release more effectively than extracts from unfertilized garlic (**Fig. 2A, 2B**). Treatment with the garlic metabolite DADS (1-100 μM) reduced also significantly both, TNF-α and IL-1β levels in human whole blood. Allicin, the major degradation product of alliin showed no effect on TNF-α and IL-1β levels up to a concentration of 100 μM but significantly inhibited the release of IL-10 at 100 μM in human whole blood. AMSO₂ and γ-glutamylcysteine had neither an effect on TNF-α nor on IL-1β or IL-10 levels (Keiss 2003; Keiss *et al.* 2003). NF-κB activity, subsequently measured in HEK293 cells transfected with a NF-κB-driven luciferase gene, was indeed significantly lower in cells exposed to blood sample supernatants treated with garlic powder extract and LPS compared to cells exposed to supernatants activated with LPS only (**Fig. 2C**) (Keiss 2003; Keiss *et al.* 2003). Further studies
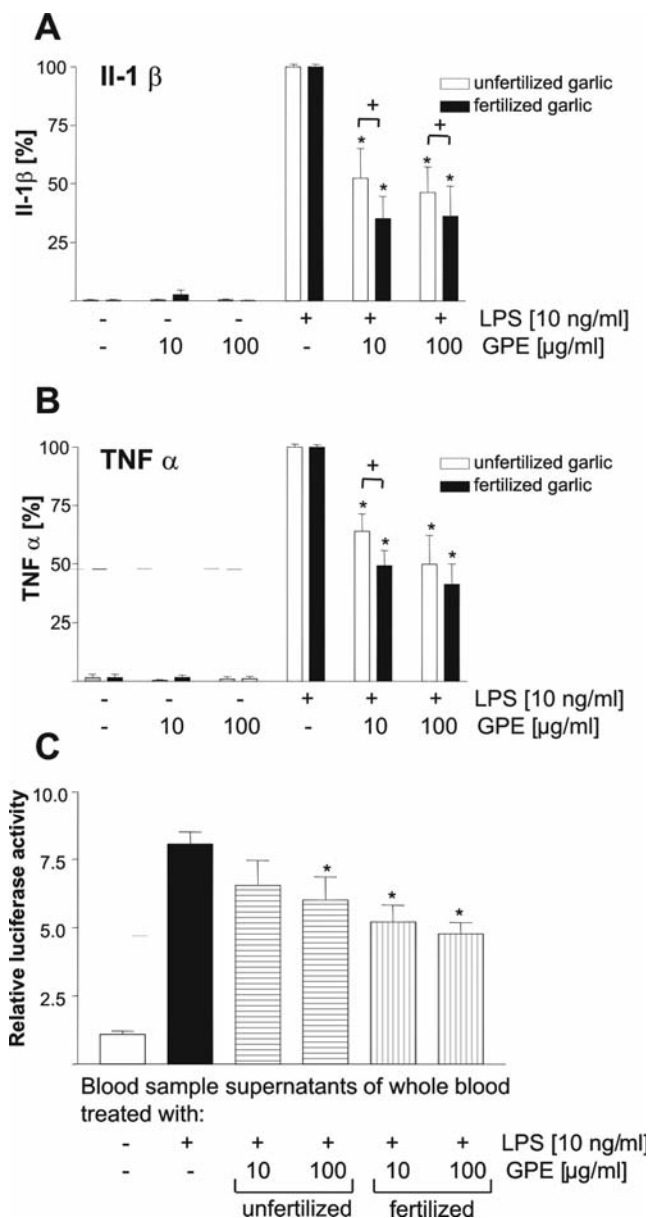


**Fig. 2 Garlic powder extracts (GPE) inhibit lipopolysaccharide (LPS)-induced liberation of Interleukin (IL)-1β (A) and tumor necrosis factor (TNF)-α (B) in human whole blood.** The mean amount of liberated IL-1β (15.7 ± 5.1 ng/ml) and TNF-α (8.8 ± 2.4 ng/ml) in LPS-treated blood samples was set as 100%. Bars represent the mean ± SEM, n=4.* Different from LPS-activated cells, p<0.05. + Different from unfertilized garlic, p<0.05. (**C**) Garlic powder extract (GPE)-treated blood samples reduce NF-κB activity in HEK293 cells. Cells were incubated for 6 h with 5 μl of cell-free supernatant from whole blood samples treated as indicated. NF-κB activity of HEK 293 cells stimulated with untreated blood supernatants was set as 1. Bars represent the mean ± SEM, n=3. * Different from LPS-activated cells, p<0.05. From: **Keiss** *et al.* (2003), with kind permission from the American Society for Nutrition, Bethesda, MD, USA.

by other groups addressing the modulation of cytokine production by garlic employed different model systems but basically agreed in the outcome that garlic affects cytokine production in a way that may result in an anti-inflammatory response (Lang *et al.* 2004; Makris *et al.* 2005).

## HUMAN STUDIES

A number of studies focussing on antiatherosclerotic effects of garlic in humans were published by different laboratories during the period of the Garlic & Health project and thereafter. In general, they all differ considerably in study design, number and type of patients, as well as type, quality and characterization of the garlic material. While some of them

reported on positive effects (Koscielny *et al.* 1999), others did not find clinically relevant effects either on lipid profiles and/or on the atherosclerotic process (Berthold and Sudhop 1998; Superko and Krauss 2000; Gardner *et al.* 2001; Turner *et al.* 2004).

Since animal studies failed to indicate that garlic constituents or well-characterized garlic powders may influence lipid profiles and atherosclerotic lesions (see above), the primary focus of a human intervention study within the Garlic & Heath consortium was placed on biomarkers for inflammation and endothelial function, but also on lipid metabolism in subjects with risk factors for CVD (van Doorn *et al.* 2006). In order to contribute in a thorough way to the discussion about garlic efficacy a double-blind, randomized placebo-controlled trial was performed using chemically well-characterized garlic powder (2.1 g/day) and comparing its effect with that of atorvastatin (40 mg/day) and placebo. Each group consisted of 30 randomly assigned subjects of overweight (BMI>24.5 kg/m²) smokers (>10 cigarettes/day). None of the variables measured showed significant changes between the garlic *versus* placebo group. In contrast, atorvastatin resulted in significantly lower plasma concentrations of C-reactive protein, total cholesterol, LDL-cholesterol, triglycerides, TNF-alpha, and other inflammatory markers (van Doorn *et al.* 2006). This well-conducted clinical trial, therefore, does not support beneficial effects of garlic in normolipidemic subjects with risk factors for cardiovascular disease.

## CONCLUDING REMARKS

Investigations within the Garlic & Health consortium on garlic efficacy with respect to CVD provided compelling evidence against a beneficial influence of garlic powders or garlic constituents on risk factors and pathological aspects of this disease in animals and humans. As discussed herein, these findings are in concert with the majority of other *in vivo* studies performed in this field. Even though part of the experimental work done *in vitro* points to some chances for defined effects of garlic components on cardio-vascular and inflammatory parameters, it remains doubtful whether these effects may be of any significance with respect to the human situation.

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