Aus dem Institut für Ernährungswissenschaften

(Institutsdirektor: Prof. Dr. habil. K. Eder)

der Landwirtschaftlichen Fakultät

(Dekan: Prof. Dr. habil. W. Merbach)

der

Martin-Luther-Universität

Halle-Wittenberg

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**Nutritive Beeinflussung der Oxysterolkonzentrationen durch Fischöl, Vitamin E, Eisen und oxidiertes Cholesterin sowie ausgewählte physiologische Wirkungen oxidierten Cholesterins bei der Ratte**

# DISSERTATION

zur Erlangung des akademischen Grades
Doktor der Ernährungswissenschaften (Dr. troph.)

von Diplom-Ökotrophologe
Robert Ringseis

Halle/Saale 2003

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**Abkürzungsverzeichnis**

| | |
|---|---|
| ACAT | Acyl-CoA:Cholesterol-Acyltransferase |
| AIN | American Institute of Nutrition |
| α-TÄ | α-Tocopherol-Äquivalente (1 mg α-TÄ = 1 mg RRR α-Tocopherol) |
| CoA | Coenzym A |
| Cu/Zn-SOD | Kupfer/Zink-haltige Superoxiddismutase |
| CYP | Cytochrom P450 |
| CYP7A1 | Cholesterol-7α-Hydroxylase |
| DGE | Deutsche Gesellschaft für Ernährung |
| GC-MS | Gaschromatographie-Massenspektrometrie |
| GSH | Glutathion |
| GSH-Px | Glutathionperoxidase |
| G-6-PDH | Glukose-6-phosphat-Dehydrogenase |
| HDL | Lipoprotein hoher Dichte (high density lipoprotein) |
| HMG-CoA-Reduktase | β-Hydroxy-β-methylglutaryl-CoA-Reduktase |
| $H_2O_2$ | Wasserstoffperoxid |
| ICAM | intercellular adhesion molecule |
| IE | Internationale Einheiten (1 IE Vitamin E = 0,67 mg α-TÄ = 0,67 mg RRR-α-Tocopherol = 1 mg all-*rac*-α-Tocopherylacetat |
| LDL | Lipoprotein geringer Dichte (low density lipoprotein) |
| LXRα | liver X receptor alpha |
| mRNA | messenger ribonucleic acid |
| MTP | Mikrosomales Triglycerid-Transferprotein |
| MUFA | einfach ungesättigte Fettsäuren (monounsaturated fatty acids) |
| n-3-Fettsäure | omega-3-Fettsäure |
| n-6-Fettsäure | omega-6-Fettsäure |
| NaCl | Natriumchlorid |
| P | Signifikanzniveau |
| PC | Phosphatidylcholin |
| PE | Phosphatidylethanolamin |

| | |
|---|---|
| PUFA | mehrfach ungesättigte Fettsäuren (polyunsaturated fatty acids) |
| ROS | reaktive Sauerstoffspezies (reactive oxygen species) |
| SFA | gesättigte Fettsäuren (saturated fatty acids) |
| SOD | Superoxiddismutase |
| SRE | sterol regulatory element, sterol responsive element |
| SREBP | sterol regulatory element binding protein |
| TBA | Thiobarbitursäure (thiobarbituric acid) |
| TBARS | Thiobarbitursäure-reaktive Substanzen (thiobarbituric acid reactive substances) |
| U | Unit |
| VCAM | vascular cell adhesion molecule |
| VLDL | Lipoprotein sehr geringer Dichte (very low density lipoprotein) |
| 7α-OH-Chol | 7α-Hydroxycholesterol |
| 7β-OH-Chol | 7β-Hydroxycholesterol |
| 7β-OOH-Chol | 7β-Hydroperoxycholesterol |
| 7-Keto-Chol | 7-Ketocholesterol |
| 7-OOH-Chol | 7-Hydroperoxycholesterol |

## 1.      Einleitung

Cholesteroloxidationsprodukte oder Oxysterole sind oxygenierte Cholesterolderivate, die neben der Hydroxylgruppe am C-Atom 3 ein weiteres Sauerstoffatom in Form einer Carbonyl-, Hydroxyl- oder Epoxidgruppe am Ring A oder B oder an der Seitenkette des Cholesterols aufweisen. Oxysterole werden im Organismus entweder endogen gebildet oder werden exogen über die Nahrung aufgenommen. Die endogene Bildung von Oxysterolen im Organismus erfolgt einerseits auf enzymatischem Wege durch Oxygenasen der Cytochrom P450-Familie – kurz CYP-Enzyme genannt - oder durch Nichthämeisen-Enzyme wie der Cholesterol-25-Hydroxylase (Abbildung 1). Enzymatisch gebildete Oxysterole spielen eine wichtige Rolle in der Cholesterolhomöostase; sie treten als Intermediate bei der Gallensäurensynthese auf und fungieren als Ausscheidungs- bzw. Transportform des Cholesterols aus extrahepatischen Geweben (BJÖRKHEM et al. 1997, BJÖRKHEM 2002, BJÖRKHEM et al. 2002, MEANEY et al. 2001, PRINCEN et al. 1997).
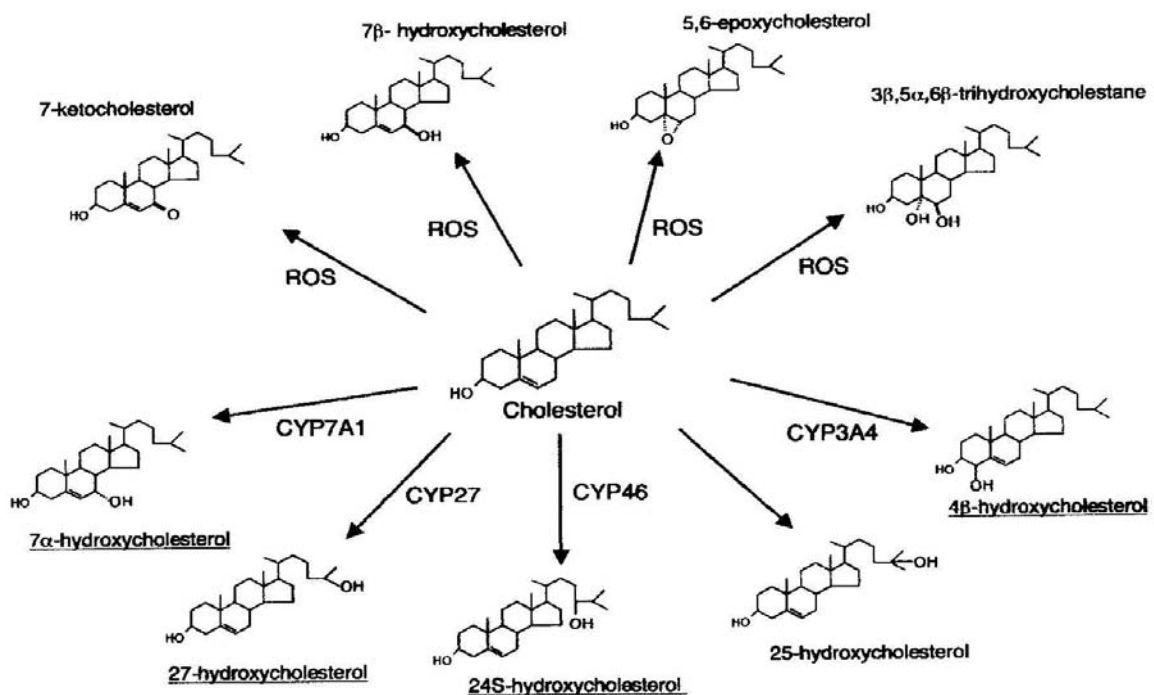


**Abb. 1:** Bedeutende Oxysterole im Humanplasma (quantitativ dominierende Vertreter sind unterstrichen). (aus BJÖRKHEM et al. 2002)

Andererseits entstehen Oxysterole im Organismus durch direkte Einwirkung von reaktiven Sauerstoffspezies (ROS) (SMITH 1996), insbesondere aber als Folge der Peroxidation polyungesättigter Fettsäuren (polyunsaturated fatty acids - PUFA) (SEVANIAN und

MCLEOD 1987). Deshalb werden insbesondere 7β-Hydroxycholesterol (7β-OH-Chol) aber auch 7-Ketocholesterol (7-Keto-Chol), Cholestanetriol (= 3β,5α,6β-trihydroxycholestane) und die epimeren Cholesterol-5,6-epoxide als *in vivo* Parameter der Lipidperoxidation bzw. als Marker für oxidativen Stress angewendet (ADACHI et al. 2001, HODIS et al. 2000, ISHII et al. 2002, IULIANO et al. 2001 und 2003, PORKKALA-SARATAHO et al. 2000, SALONEN et al. 1997, SKRZEP-POLOCZEK et al. 2001, ZIEDEN et al. 1999). Die Bildung dieser Oxysterole wird in der Literatur überwiegend als „nicht-enzymatisch" bezeichnet, obwohl die enzymatische Konversion von 7-Keto-Chol zu 7β-OH-Chol in Rattenlebermikrosomen mehrfach beschrieben wurde (BJÖRKHEM et al. 1968, BREUER et al. 1993, MENDELSOHN et al. 1965). Ferner wurde gezeigt, dass die membranständige Phospholipidhydroperoxid-Glutathionperoxidase zumindest *in vitro* nicht nur Phospholipidhydroperoxide, sondern auch freie und veresterte 7-Hydroperoxycholesterole detoxifizieren kann (KORYTOWSKI et al. 1996, THOMAS et al. 1990). Diese Reaktion spielt vermutlich auch *in vivo* im oxidativen Stress eine Rolle, wenn intrazellulär große Mengen an Cholesterol- und anderen Lipidhydroperoxiden anfallen (ISHII et al. 2002). Die enzymatische Reduzierung dieser Lipidhydroperoxide zu weniger toxischen Alkoholen ist ein Schutzmechanismus der Zellen vor peroxidativen Schädigungen (URSINI et al. 1991).

Eine gesteigerte Lipidperoxidation wird unter anderem in Zusammenhang mit der Entstehung der Atherosklerose diskutiert (HALLIWELL und GUTTERIDGE 1989). So konnten im oxidativ modifizierten *low density lipoprotein* (LDL), welches aufgrund seiner Zytotoxizität eine zentrale Rolle in der Atherogenese aufweist (CATHCART et al. 1985, HESSLER et al. 1983, KOSUGI et al. 1987, MOREL et al. 1983, REID and MITCHINSON 1993, SEVANIAN et al. 1995), im Rahmen der Lipidperoxidation gebildete Oxysterole nachgewiesen werden (BROWN et al. 1996 und 1997, HODIS et al. 1994, CARUSO et al. 1996, MALAVASI et al. 1992, MORI et al. 1996, SEVANIAN et al. 1995). Auch in frühen und fortgeschrittenen atheromatösen Läsionen konnten diese Oxysterole in hohen Konzentrationen nachgewiesen werden (BROWN et al. 1997, CARPENTER et al. 1993 und 1995, GARCIA-CRUSET et al. 1999 und 2001, VAYA et al. 2001). Diese Ergebnisse sind ein Hinweis auf verstärkt ablaufende oxidative Prozesse, die mutmaßlich in der Atherogenese involviert sind (AVIRAM 2000, IULIANO 2001, RUEF et al. 1999), erklären jedoch nicht die Atherogenität dieser Oxysterole. Der Befund, dass 7β-OH-Chol und 7-Keto-Chol, neben dem Intermediat 7β-Hydroperoxycholesterol, als die Komponenten mit der höchsten Toxizität im oxidierten LDL identifiziert wurden (CHISOLM et al. 1994, HUGHES et al. 1994), deuten

auf eine mögliche Atherogenität dieser Oxysterole hin. Die proatherogene Wirkung von 7β-OH-Chol und 7-Keto-Chol erklärt sich möglicherweise dadurch, dass diese Oxysterole zelltoxisch und/oder Apoptose-induzierend auf die Hauptzelltypen der Arterienwand wie Endothelzellen, glatte Muskelzellen und Fibroblasten, aber auch auf Monocyten bzw. Macrophagen wirken (BANSAL et al. 2001, LEMAIRE et al. 1998, LIZARD et al. 1996, 1997 und 1999, LYONS et al. 2001, MIGUET-ALFONSI et al. 2002, O´CALLAGHAN et al. 1999 und 2002). Auch führen diese Oxysterole zu einer erhöhten Sekretion von Interleukin-1β und zu einer erhöhten endothelialen Expression verschiedener Adhäsionsmoleküle (ICAM-1, VCAM-1, E-Selektin) (LEMAIRE et al. 1998, LIZARD et al. 1997), die in der frühen Phase der Atherogenese verstärkt exprimiert werden und den Kontakt zwischen immunkompetenten Zellen (Monozyten, T-Lymphozyten) und dem Gefäßendothel vermitteln (BERLINER und HEINECKE 1996, SPRINGER 1994). Humanstudien konnten belegen, dass erhöhte Plasmakonzentrationen an 7β-OH-Chol auf ein erhöhtes Atheroskleroserisiko und eine erhöhte *in vivo* Lipidperoxidation hindeuten (SALONEN et al. 1997, ZIEDEN et al. 1999). Auch Befunde, dass 7β-OH-Chol und andere „nicht-enzymatisch" gebildete Oxysterole erst bei Konzentrationen zytotoxisch wirksam sind, die im Plasma von Stenosepatienten bzw. Hypercholesterolämikern und in atherosklerotischen Läsionen gemessen wurden (BANSAL et al. 2001, PETTERSEN et al. 1991, ZHOU et al. 2000), deuten an, dass erhöhte Konzentrationen an den durch ROS-Einwirkung formierten Oxysterolen ein Gefährdungspotential für die Gesundheit des Organismus darstellen. Dies wird auch anhand der Ergebnisse einer neueren Studie deutlich, dass die Plasmakonzentrationen an 7β-OH-Chol auch mit dem Lungenkrebsrisiko positiv assoziiert sind (LINSEISEN et al. 2002). LINSEISEN et al. (2002) schlagen aufgrunddessen auch die Verwendung diesen Parameters als Biomarker für die Vorhersage des Lungenkrebsrisikos als möglicherweise geeignet vor.

In Anbetracht der pathophysiologischen Bedeutung spezifischer „nicht-enzymatisch" gebildeter Oxysterole, insbesondere des 7β-OH-Chol, stellt sich aus ernährungswissenschaftlicher Sicht die Frage, ob die Konzentrationen dieser Oxysterole in relevanten Geweben durch die Ernährung beeinflusst werden können. Es ist hinreichend bekannt, dass sich über die Diätzusammensetzung oxidativer Stress bzw. die Bildung von ROS im Organismus induzieren lässt, was zu einem starken Anstieg der Lipidperoxidation führt (CHEN et al. 1993, CHO und CHOI 1994, HU et al. 1989, JAVOUHEY-DONZEL et al. 1993, KUBOW et al. 1996, LEIBOWITZ et al. 1990). Da 7β-OH-Chol und andere „nicht-

enzymatisch" gebildete Oxysterole sekundär als Folge der Peroxidation von PUFAs entstehen (SEVANIAN and MCLEOD 1987, TERAO et al. 1985), dürften prooxidativ wirksame Nährstoffe bzw. Nahrungskomponenten die Bildung dieser Oxysterole begünstigen, während antioxidativ wirksame Nahrungsinhaltsstoffe diese hemmen dürften. Anhaltspunkte hierfür liefern tierexperimentelle Arbeiten zur Erforschung der toxischen Wirkung von Alkohol. Bei Verfütterung einer Ethanol-haltigen Diät an Ratten kam es zu einem starken Konzentrationsanstieg an 7β-OH-Chol in Herz- (ADACHI et al. 2001), Leber- (ARIYOSHI et al. 2002) und Skelettmuskelgewebe (FUJITA et al. 2002). Dies wird auf eine gesteigerte Lipidperoxidation zurückgeführt, die als Ursache der Alkoholtoxizität bei Alkoholischer Myopathie und Kardiomyopathie gilt (JAATINEN et al. 1993, MANTLE und PREEDY 1999). Ebenfalls konnte nachgewiesen werden, dass eine Cholesterol-reiche Diät die Lipidperoxidation steigert (BULUR et al. 1986, SULYOK et al. 1984) und zu einem Konzentrationsanstieg an 7β-OH-Chol im Plasma von Ratten bzw. Kaninchen führt (HODIS et al. 1991, MAHFOUZ et al. 1997, MAHFOUZ und KUMMEROW 2000, VINE at al. 1998). Auch gibt es einige Untersuchungen zur Wirkung von Antioxidantien wie Vitamin E, Vitamin C, Probucol, Butyliertes Hydroxytoluen auf die *in vivo*-Bildung von Oxysterolen mit allerdings nicht einheitlicher Wirkung (BJÖRKHEM et al. 1991, HODIS et al. 1992, IULIANO et al. 2001, PORKKALA-SARATAHO et al. 2000, MAHFOUZ et al. 1997, MOL et al. 1997, UPSTON et al. 2001, WISEMAN et al. 1995).

Die Induzierung von oxidativem Stress ist insbesondere durch eine hohe Aufnahme langkettiger n-3-PUFA möglich, die in **Fischöl**en in hohen Konzentrationen enthalten sind. Dieser Befund wurde bereits durch eine Vielzahl an Studien belegt (CHO und CHOI 1994, D`AQUINO et al. 1991, HU et al. 1989, IBRAHIM et al. 1997, KUBOW et al. 1996, LEIBOWITZ et al. 1990, MEYDANI et al. 1987, MOURI et al. 1984). *In vitro*-Studien an Membranlipiden lieferten Hinweise, dass bestimmte Oxysterole sekundär als Folge der Peroxidation oxidationsempfindlicher n-3-PUFA entstehen (SEVANIAN and MCLEOD 1987, TERAO et al. 1985). Es ist deshalb auch anzunehmen, dass die Inkorporierung von PUFAs nach Aufnahme von Fischöl zu einer verstärkten Bildung von Oxysterolen nicht-enzymatischen Ursprungs *in vivo* führt. Allerdings sind aus der Literatur diesbezügliche Studien bislang nicht bekannt.

Auch katalytisch wirksames **Eisen** führt im Organismus zu oxidativem Stress, indem es über die Bildung von ROS die Lipidperoxidation (CROSS et al. 1987, HALLIWELL und GUTTERIDGE 1986 und 1990), aber auch die Degradation von Proteinen und Nukleinsäuren

fördert (AUST et al. 1986, GLEI et al. 2002, HALLIWELL und GUTTERIDGE 1986, MINOTTI 1993, TOYOKUNI 1996). Im Zustand der Eisenüberladung wird die Eisenbindungskapazität des Organismus überschritten, so dass freie Eisenionen die Bildung freier Radikale, insbesondere hochreaktiver Hydroxylradikale über die Fenton-Reaktion, katalysieren (HALLIWELL und GUTTERIDGE 1990). Eisenüberladung tritt unter anderem bei Patienten mit Acoeruloplasminämie, mit der Folge einer starken Eisenanreicherung in Retina und Gehirn und einer erhöhten Lipidperoxidation auf. Neben erhöhten Konzentrationen an Thiobarbitursäure-reaktiven Substanzen (TBARS) und/oder Malondialdehyd in Serum, Cerebrospinalflüssigkeit und/oder Gehirn (MIYAJIMA et al. 1996 und 1998, YOSHIDA et al. 2000) wurden bei diesen Patienten auch erhöhte Oxysterolkonzentrationen (7β-OH-Chol und 7-Keto-Chol) in Gehirn und anderen Organen festgestellt, die positiv mit der Konzentration an akkumulierten Eisen korrelierten (MIYAJIMA et al. 2001). Es stellt sich deshalb die Frage, ob eine hohe Eisenaufnahme mit der Nahrung über eine gesteigerte Lipidperoxidation die Konzentrationen nicht-enzymatisch gebildeter Oxysterole erhöht. Obwohl einige Studien existieren, die den Zusammenhang zwischen hoher Eisenzufuhr und oxidativen Schädigungen untersuchen (BACON et al. 1983a und 1983b, DABBAGH et al. 1994, DILLARD et al. 1983, WU et al. 1990), ist der Effekt einer hohen Eisenzufuhr auf die Bildung von Oxysterolen in der Literatur bislang nicht beschrieben.

Antioxidantien wie **Vitamin E** sind Substanzen, die ROS bzw. freie Radikale unschädlich machen und vor oxidativen Schädigungen schützen (BURTON und TRABER 1990, BURTON und INGOLD 1986). Das in den Membranen lokalisierte, lipidlösliche Vitamin E bewirkt den Abbruch von Radikalkettenreaktionen, wie sie bei der Oxidation von Fettsäuren auftreten, und verhindert dadurch die weitere Reaktion von Fettsäureradikalen mit Membranbestandteilen wie Phospholipiden, Proteinen und Cholesterol. Es ist deshalb anzunehmen, dass eine hohe Zufuhr an Vitamin E die Bildung von „nicht-enzymatisch" gebildeten Oxysterolen vermindert. In Humanstudien führte Vitamin E-Supplementierung zu einem Rückgang der Plasmakonzentrationen an 7β-OH-Chol bei Gesunden (PORKKALA-SARATAHO et al. 2000) bzw. an 7β-OH-Chol, 7-Keto-Chol und Cholestanetriol bei Diabetikern (MOL et al. 1997). Dagegen kam es bei gesunden Probanden in den Studien von MOL et al. (1997) und IULIANO et al. (2001) zu keiner signifikanten Verringerung an 7β-OH-Chol und 7-Keto-Chol. Bei Kaninchen führte Vitamin E in einer Studie zu einer Reduzierung der Plasma-Oxysterolkonzentrationen (UPSTON et al. 2001), während dies in einer anderen Studie nicht der Fall war (WISEMAN et al. 1995). Bei genauerer Betrachtung

des Studiendesigns wird allerdings deutlich, dass die Widersprüchlichkeit der Ergebnisse vor allem aus Unterschieden im Vitamin E-Status zu Beginn der Supplementierungsphase zwischen den Studien resultiert. Da der Vitamin E-Bedarf auch in hohem Maße von der Zufuhr an PUFA beeinflusst wird (MUGGLI 1994), lässt sich vermuten, dass die Bildung von „nicht-enzymatisch" gebildeten Oxysterolen bei Zufuhr eines n-3-PUFA-reichen Fettes größer ist als bei Zufuhr eines n-3-PUFA-armen Fettes unter der Voraussetzung, dass beide Diäten die gleiche Vitamin E-Konzentration aufweisen. In der Literatur sind diesbezügliche Interaktionen allerdings bislang nicht beschrieben.

Auch die Aufnahme **oxidierten Cholesterols**, das Bestandteil tierischer Lebensmittel wie Fleisch, Wurst, Milchprodukte, aber auch Ei-haltiger Backwaren und Säuglingsnahrungen ist (PANIANGVAIT et al. 1995, PRZYGONSKI et al. 2000, ZUNIN et al. 1995), beeinflusst die Oxysterolkonzentrationen im Organismus. Dafür ist einerseits die Absorption der Oxysterole aus dem Darm (EMANUEL et al. 1991, LINSEISEN und WOLFRAM 1998a, TOMOYORI et al. 2002) sowie deren Inkorporierung in Chylomikronen (OSADA et al. 1994a, VINE et al. 1997) und deren Transport zur Leber verantwortlich. Auch werden diese Oxysterole in Lipoproteine sehr geringer Dichte (very low density lipoprotein-VLDL) inkorporiert (LINSEISEN und WOLFRAM 1998a, VINE et al. 1998) und gelangen somit in die Zirkulation. Andererseits existieren auch Hinweise, dass Oxysterole bzw. oxidiertes Cholesterin per se prooxidativ wirken und somit die „nicht-enzymatische" Oxysterolbildung *in vivo* induzieren (BANSAL et al. 2001, BLACHE et al. 1995, CANTWELL and DEVERY 1998, LIZARD et al. 2000, LYONS et al. 2001, MCCLUSKEY et al. 1999, MIGUET-ALFONSI et al. 2002, OSADA et al. 1995, 1998 und 1999, WILSON et al. 1997). Dies würde bedeuten, dass Vitamin E aufgrund seiner antioxidativen Eigenschaften den prooxidativen Effekt der Oxysterole kompensiert und deren *in vivo* Bildung vermindert. Auch derartige Interaktionen sind bislang kaum untersucht.

Eine Vielzahl an Studien haben die biologischen Wirkungen spezifischer Oxysterole, die in Zusammenhang mit pathologischen Prozessen (Atherogenese, Kanzerogenese, Mutagenese) diskutiert werden, zum Forschungsgegenstand (GUARDIOLA et al. 1996, MOREL und LIN 1996). Die meisten Beobachtungen stammen jedoch aus *in vitro*-Studien, in denen die Wirkung isoliert verabreichter Oxysterole untersucht wurde. Diese Untersuchungen entsprechen nicht der *in vivo*-Situation, da unberücksichtigt bleibt, dass Oxysterole in Biomembranen und Lipoproteinen in Verbindung mit anderen Oxysterolen und darüber hinaus nur im $10^3$- bis $10^6$-fachen Überschuss mit Cholesterin vorkommen (BJÖRKHEM und

DICZFALUSY 2002). Oxysterole werden über mehr oder weniger stark oxidiertes Cholesterin aufgenommen, welches Bestandteil tierischer, Cholesterin-haltiger Lebensmittel ist. Deshalb ist die Verwendung oxidierten Cholesterins als Oxysterolquelle relevanter für die Übertragbarkeit der Ergebnisse auf die Humanernährung als die Verwendung isolierter hochkonzentrierter Oxysterole. *In vitro*-Studien lieferten Hinweise, dass Oxysterole wie $7\beta$-OH-Chol und 7-Keto-Chol oxidativen Stress induzieren (BANSAL et al. 2001, CANTWELL und DEVERY 1998, LIZARD et al. 2000, LYONS et al. 2001, MCCLUSKEY et al. 1999, O´CALLAGHAN et al. 2002). Da oxidativer Stress als Ursache einer gesteigerten Lipidperoxidation möglicherweise in der Atherosklerose beteiligt ist (HALLIWELL und GUTTERIDGE 1989), stellt sich die Frage, ob auch **oxidiertes Cholesterin** im Vergleich zu reinem Cholesterin bestimmte Parameter des **antioxidativen Schutzsystems** *in vivo* beeinflusst, zumal diesbezüglich nur wenige Informationen aus der Literatur bekannt sind. So konnte bei vergleichenden Untersuchungen zwischen reinem und oxidiertem Cholesterin gezeigt werden, dass oxidiertes Cholesterin zu erhöhten TBARS-Konzentrationen in Leber und Blut von Ratten führt (OSADA et al. 1995, 1998 und 1999), was auf die Induzierung von oxidativem Stress durch oxidiertes Cholesterin schliessen lässt, aber keine eindeutigen Rückschlüsse auf die Modulierung antioxidativ wirksamer Parameter zulässt.

Vergleichende Studien zwischen reinem und oxidiertem Cholesterin ergaben ferner, dass oxidiertes Cholesterin den Lipidstoffwechsel bei verschiedenen Spezies beeinflusst (OSADA et al. 1994b, 1995, 1996 und 1998, VINE et al. 1998). Im Tierversuch führte oxidiertes Cholesterin unter anderem zu einer erhöhten Desaturation der Linolsäure und zu einer Beeinflussung des Cholesterolmetabolismus, was unter anderem auf eine Aktivitätshemmung der Cholesterol-$7\alpha$-Hydroxylase (CYP7A1) und der $\beta$-Hydroxy-$\beta$-Methylglutaryl-CoA-Reduktase (HMG-CoA-Reduktase) und eine Aktivitätssteigerung der Acyl-CoA:Cholesterol-Acyltransferase (ACAT) zurückgeführt wird (OSADA et al. 1998 und 1999, SMITH und JOHNSON 1989, VARGAS et al. 1986). Es zeigte sich auch, dass oxidiertes im Vergleich zu reinem Cholesterol zu verminderten Triglycerid- und/oder Cholesterolkonzentrationen im Plasma führt (CHO et al. 1986, OSADA et al. 1994b und 1999, VINE et al. 1998), was möglicherweise auf eine Beeinflussung der VLDL-Sekretion bzw. –Synthese hindeutet. Da bislang kaum Hinweise auf die Ursache der reduzierten Plasmalipidkonzentrationen nach Aufnahme oxidierten Cholesterins existieren, wurde auch die Fragestellung untersucht, ob **oxidiertes Cholesterin** im Vergleich zu reinem Cholesterin Parameter moduliert, die einen Einfluss auf die **VLDL-Synthese und –Sekretion** ausüben.

## 2.      Zielstellung

Aufgrund der pathophysiologischen Bedeutung spezifischer Oxysterole für die Gesundheit des menschlichen und tierischen Organismus und der Möglichkeit der nutritiven Beeinflussung der endogenen Oxysterolbildung war es Ziel dieser Arbeit zu untersuchen, ob verschiedene pro- oder antioxidativ wirksame Nahrungskomponenten bzw. Nährstoffe (Fischöl, Eisen, oxidiertes Cholesterin, Vitamin E) die Konzentrationen an bestimmten Oxysterolen beeinflussen. Diese Untersuchungen wurden mit der Ratte als Modelltier durchgeführt, da es sich in einer Vielzahl an Untersuchungen als geeignetes *in vivo*-Modell zur Induktion von oxidativem Stress und/oder zur Untersuchung der *in vivo*-Bildung von Oxysterolen erwiesen hat (ADACHI et al. 2001, ARIYOSHI et al. 2002, BACON et al. 1983a und 1983b, DABBAGH et al. 1994, DILLARD et al. 1983, FUJITA et al. 2002, MAGGI-CAPEYRON et al. 2002, MAHFOUZ und KUMMEROW 2000, WU et al. 1990). Ferner wurden die Wirkungen oxidierten Cholesterins auf ausgewählte Parameter des antioxidativen Schutzsystems und des Lipidstoffwechsels der Ratte untersucht.

(1)      Zielstellung der ersten zweifaktoriell angelegten Studie war, zu untersuchen, ob hohe Eisendosen in der Diät von Ratten das antioxidative Schutzsystem und Parameter der Lipidperoxidation (TBARS, spezifische Oxysterole) in Abhängigkeit vom Diätfett beeinflussen. Mittels diesen Versuches wurde auch die Fragestellung untersucht, ob die Oxysterolkonzentrationen in der Leber durch die Fettart und die Höhe der Eisenkonzentration in der Diät beeinflusst werden. Als Diätfette wurden Lachsöl, als Vertreter eines n-3 PUFA-reichen Fettes, und Schweineschmalz, als ein Vertreter eines n-3 PUFA-armen Fettes, verwendet. Die Eisenkonzentrationen in den Diäten wurden in den zwei Stufen ausreichend (50 mg Eisen/kg Diät) und hoch (500 mg Eisen/kg Diät) variiert. Ferner sollte ermittelt werden, ob Interaktionen bezüglich der Oxysterolkonzentrationen in der Leber zwischen der Fettart und der Höhe der Eisenzufuhr bestehen. Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

*Brandsch C, Ringseis R, Eder K (2002) High dietary iron concentrations enhance the formation of cholesterol oxidation products in the liver of adult rats fed salmon oil with minimal effects on antioxidant status.* ***Journal of Nutrition*** *132:2263-2269.*

(2) Die Zielstellung der zweiten Studie bestand darin, zu untersuchen, ob die Konzentrationen an 7β-OH-Chol durch oxidiertes Cholesterol und Vitamin E in relevanten Geweben von Ratten beeinflusst werden. Da auch die Art des Fettes einen Einfluss haben dürfte, wurde der Versuch mit zwei verschiedenen Diätfetten (Kokosfett und Lachsöl) durchgeführt. Aufgrund der großen Zahl an Versuchsfaktoren und der sich daraus ergebenden möglichen Interaktionen wurde die Studie in zwei getrennten Versuchsmodellen parallel durchgeführt. Im ersten zweifaktoriell angelegten Versuchsansatz wurden nur die Faktoren Diätfett (Fischöl vs. Kokosfett) und Vitamin E-Konzentration der Diät (40 vs. 240 mg α-Tocopheroläquivalente/kg Diät) variiert, im zweiten dreifaktoriell angelegten Versuchsansatz wurden die Faktoren Cholesterol (reines Cholesterol vs. oxidiertes Cholesterol), Diätfett (Fischöl vs. Kokosfett) und Vitamin E-Konzentration der Diät (40 vs. 240 mg α-Tocopheroläquivalente/kg Diät) variiert. Die Auswertung des zweiten dreifaktoriell angelegten Versuches erfolgte aus Übersichtlichkeitsgründen zweifaktoriell, d.h. nach den Faktoren Cholesterol und Vitamin E jeweils getrennt für die Tiere, die Lachsöl erhielten, und für die Tiere, die Kokosfett erhielten. Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

*Ringseis R, Eder K (2003) Effects of dietary fish oil and oxidized cholesterol on the concentration of 7β-hydroxycholesterol in liver, plasma, low density-lipoprotein and erythrocytes of rats at various vitamin E supply.* **European Journal of Lipid Science and Technology.** (im Druck)

(3) Die dritte Studie verfolgte als Zielstellung, die Wirkung des Vitamin E auf die *in vivo* Bildung von 7β-OH-Chol in Abhängigkeit des Diätfettes genauer zu untersuchen. Hierzu wurde ein zweifaktorielles Versuchsmodell gewählt, in dem die Höhe der Vitamin E-Konzentration und das Diätfett variiert wurden. Die Vitamin E Konzentrationen der Diäten wurden so gewählt, dass der Bereich von unzureichend über knapp und optimal bis hin zu exzessiv abgedeckt wurden. Somit ergaben sich folgende Vitamin E-Konzentrationen: 10, 20, 40 und 240 mg α-Tocopheroläquivalente/kg Diät. Als Diätfette wurden wiederum zwei Fette gewählt, die sich insbesondere in ihrem Gehalt an n-3-PUFAs unterschieden (Fischöl versus

Kokosfett). Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

*Ringseis R, Eder K (2002) Insufficient dietary vitamin E increases the concentration of 7β-hydroxycholesterol in tissues of rats fed salmon oil.* **Journal of Nutrition 132:3732-3735.**

(4)     Eine vierte Studie untersuchte die Effekte oxidierten Cholesterols im Vergleich zu reinem Cholesterol auf Parameter des antioxidativen Schutzsystems von Ratten bei Verabreichung unterschiedlicher Diätfette. Es wurde ein zweifaktorielles Versuchsmodell gewählt. Die Versuchsfaktoren Cholesterol (reines Cholesterol vs. oxidiertes Cholesterol) und Diätfett (Fischöl vs. Kokosfett) sowie die Interaktion dieser beiden Faktoren wurden untersucht. Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

*Ringseis R, Eder K: Effects of dietary oxidized cholesterol on the antioxidant defence system in rats fed coconut oil or salmon oil.* **International Journal for Vitamin and Nutrition Research.** (Manuskript: eingereicht zur Publikation 2002)

(5)     In einer fünften Studie wurden die Effekte oxidierten Cholesterols im Vergleich zu reinem Cholesterol auf Lipidkonzentrationen in Plasma und Lipoproteinen sehr geringer Dichte (VLDL) sowie auf Parameter der hepatischen VLDL-Synthese von Ratten bei Verabreichung unterschiedlicher Diätfette untersucht. Es wurde ein zweifaktorielles Versuchsmodell gewählt. Die Versuchsfaktoren Cholesterol (reines Cholesterol vs. oxidiertes Cholesterol) und Diätfett (Fischöl vs. Kokosfett) sowie die Interaktion dieser beiden Faktoren wurden untersucht. Weitere Details zu Material und Methodik sowie die ausführliche Beschreibung und Diskussion der Ergebnisse dieser Studie sind ersichtlich in:

*Ringseis R, Eder K: Dietary oxidized cholesterol decreases expression of hepatic microsomal triglyceride transfer protein in rats.* **Journal of Nutritional Biochemistry.** (Manuskript: eingereicht zur Publikation 2003)

# 3.      Originalarbeiten

## 3.1      Effekt von Eisen und Fischöl auf die Oxysterolkonzentrationen in der Leber von Ratten

ABSTRACT The aim of this study was to investigate the effect of high dietary iron concentrations on the antioxidant status of rats fed two different types of fat. Four groups of male adult Sprague-Dawley rats were fed diets with adequate (50 mg iron supplemented per kg diet) or high (500 mg iron supplemented per kg diet) iron concentrations with either lard or salmon oil as dietary fat at 100 g/kg for 12 wk. The antioxidant status of the rats was profoundly influenced by the type of fat. Rats fed salmon oil diets had higher concentrations of thiobarbituric acid-reactive substances (TBARS) ($P<0.001$), various cholesterol oxidation products (COP) ($P<0.001$), total and oxidized glutathione ($P<0.05$) and a lower concentration of $\alpha$-tocopherol ($P<0.05$) in liver and plasma than rats fed lard diets. The iron concentration of the diet did not influence the concentrations of TBARS, the activities of superoxide dismutase and glutathione peroxidase or the concentration of $\alpha$-tocopherol in plasma or liver. The activity of catalase ($P<0.01$) and the concentrations of total, oxidized and reduced glutathione ($P<0.05$) in liver were slightly but significantly higher in rats fed high iron diets than in rats fed adequate iron diets, irrespective of the dietary fat. Rats fed the high iron diets with salmon oil, moreover, had higher concentrations of various COP in the liver ($P<0.001$) than rats fed adequate iron diets with salmon oil. These results suggest that feeding a high iron diet does not generally affect the antioxidant status of rats but enhances the formation of COP, particularly if the diet is rich in polyunsaturated fatty acids.

KEY WORDS: iron ● lard ● salmon oil ● antioxidant status ● cholesterol oxidation products ● rats

[2]Abbreviations used: COP, cholesterol oxidation products; FAME, fatty acid methyl esters; GSH, glutathione; GSH-Px, glutathione peroxidase; GSSG, oxidized glutathione; PUFA, polyunsaturated fatty acids; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances

Iron deficiency is a common nutritional problem (1), but there are some instances in which iron overload is considered a health issue as well. Iron overload occurs primarily in individuals with inherited disorders such as hereditary hemochromatosis, juvenile hemachromatosis, atransferrinemia and Friedreich´s ataxia (2). A high dietary intake of iron through meat or nutritional supplements is also a potential cause of iron overload. Iron promotes the generation of oxygen radicals (especially hydroxyl radicals via Fenton Chemistry), which may cause oxidative damage such as degradation of proteins and nucleic acids and peroxidation of polyunsaturated fatty acids (PUFA)[2] (3, 4). There are several animal and epidemiologic studies suggesting that a high iron status may increase the risk of heart disease (5, 6) and cancer, especially colon carcinoma (7, 8), whereas others did not confirm this (1, 9). One reason for the discrepancy between different feeding studies could be the fat used in the diet, because the effect of high dietary iron concentrations on the antioxidant system might be dependent on the type of fat. PUFA are a source of oxidative stress. The effect of high dietary iron is therefore likely to be strongest when diets with high

concentrations of PUFA are used. Little published information is available to date about the interactions between high dietary iron concentrations and different dietary fats. The present study was therefore carried out to determine the effect of high iron diets on the antioxidant status of rats fed two different dietary fats, either lard or salmon oil.

Several studies have investigated the effect of iron on lipid peroxidation (10-12). In many of these studies, the generation of thiobarbituric acid-reactive substances (TBARS) is a preferred method for estimation of lipid peroxidation. But in addition to PUFA, cholesterol may also undergo oxidative modifications. Cholesterol oxidation products (COP) have been demonstrated to possess a wide variety of biological effects and are especially implicated in the pathogenesis of atherosclerosis via oxides in LDL (13). The formation of COP may occur through enzymatic or nonenzymatic oxidation. Nonenzymatic processes include autoxidations involving several active oxygen species. It is believed that transition metal ions such as iron act as catalysts in tissue. Iron accumulation in tissues can therefore be expected to enhance the formation of COP. Studies about the relationship between the iron supply of animals and the formation of COP are lacking. Miyajima et al. (14) described enhanced concentrations of 7$\beta$-hydroxycholesterol and 7-ketocholesterol in brains and visceral organs of patients with aceruloplasminemia, a phenomenon characterized by excessive neurovisceral iron accumulation. The concentrations of these COP correlated with the amount of iron accumulated in various organs. The authors concluded that lipid peroxidation induced by the intracellular accumulation of iron is involved in the pathogenesis of aceruloplasminemia. It seems possible that raised iron levels in tissues as a result of dietary overload may also lead to enhanced lipid peroxidation and enhanced generation of COP. Our study therefore included the measurement of COP in the liver.

In general, animal studies allow standardized treatments, although many different regimens have been described. Iron overload can be achieved with different iron sources and different methods of administration. A feeding trial would be the method of choice for testing the effects of iron overload through supplementation. It is important to consider the possibility that high levels of iron may promote oxidation of the diet even before it has been fed to the animals. In preliminary studies, we prepared diets with high iron concentration (500 mg/kg diet) and PUFA-rich salmon oil (100 g/kg diet). After 7 d storage at 4°C, the diets had peroxide values >1200 mEq $O_2$/kg oil. Even when salmon oil was replaced with lard, the diets had peroxide values of ~260 mEq $O_2$/kg oil. Feeding diets with high concentrations of lipid peroxidation products has profound effects on the antioxidant system (15). Therefore, if diets with high concentrations of lipid peroxidation products are used, it is impossible to determine which effects are caused by high dietary iron concentrations per se and which are caused by dietary lipid peroxidation products. In the present study, such methodological problems were avoided by providing iron and fat in two separate dietary components.

## MATERIALS AND METHODS

*Animals.* Adult male Sprague-Dawley rats (n = 40) with an average initial body weight of 244 ± 7.7 g (mean ± SD), obtained from Charles River GmbH (Sulzfeld, Germany), were randomly assigned to one of four groups of 10 rats each. They were housed individually in Macrolon cages in a room controlled for temperature (22 ± 2°C), humidity and light (12-h light:dark cycle). All animal procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the regional council of Saxony-Anhalt.

*Diets.* According to a bifactorial experimental design, four diets were used, differing in their iron supplement and their fat (**Table 1**). Iron was supplemented at a concentration of either 50 mg/kg diet ("adequate iron diets") or 500 mg/kg diet ("high iron diets"). Ferrous sulfate was used as the iron source because it is highly bioavailable and often used in iron supplements and for food fortification. The basal iron concentration of the diet was 10 mg/kg.

The total iron concentrations of the diets were 60 and 510 mg/kg in the iron-adequate and the high iron diets, respectively. The dietary fat was either lard (obtained from O. Stiegele Schmalzsiederei, Dresden, Germany) or salmon oil (obtained from Caelo GmbH, Hilden, Germany), at a concentration of 100 g/kg diet. The fatty acid composition and the tocopherol concentrations of the fats are shown in **Table 2**. We equalized the tocopherol concentrations of the fats by supplementing them individually with all-rac-α-tocopheryl acetate to a final concentration of 400 mg α-tocopherol equivalents/kg oil (allowing for the fact that the biopotency of all-rac-α-tocopheryl acetate in rats is 67% of that of α-tocopherol (16,17)). Both types of diet contained 40 mg α-tocopherol equivalents per kg. To avoid autoxidation of dietary PUFA, fat and iron were administered in two separate diet portions, which were administered in identical amounts. The two portions combined yielded the whole diet of the rats.

**TABLE 1**
*Composition of the experimental diets fed to rats for 12 wk*

|                                        | Diet |       |            |            |
|----------------------------------------|------|-------|------------|------------|
| Dietary fat                            | Lard | Lard  | Salmon oil | Salmon oil |
| Dietary iron supplement (mg/kg)        | 50   | 500   | 50         | 500        |
| Ingredient                             | *g/kg diet* | | | |
| Casein                                 | 200  | 200   | 200        | 200        |
| Corn starch                            | 408  | 408   | 408        | 408        |
| Saccharose                             | 200  | 200   | 200        | 200        |
| Lard                                   | 100  | 100   | -          | -          |
| Salmon oil                             | -    | -     | 100        | 100        |
| Cellulose                              | 29.83 | 28.33 | 29.83     | 28.33      |
| Mineral mixture[1]                     | 40   | 40    | 40         | 40         |
| Vitamin mixture[2]                     | 20   | 20    | 20         | 20         |
| DL-Methionine                          | 2    | 2     | 2          | 2          |
| Ferrous sulfate[3]                     | 0.167 | 1.67 | 0.167      | 1.67       |

[1] Minerals supplemented (per kg diet): dicalcium phosphate, 8.92 g; calcium carbonate 8.44 g; potassium sulfate, 9.0 g; sodium chloride, 2.6 g; magnesium oxide, 166 mg; zinc oxide, 38 mg; cupric sulfate pentahydrate, 24 mg; manganous oxide, 16 mg; calcium iodate, 0.32 mg; sodium selenate, 0.33 mg.
[2] Vitamins supplemented (per kg diet): all-trans-retinol, 1.67 mg; cholecalciferol, 25 μg; menadione sodium bisulfate, 1 mg; thiamine-HCL, 5 mg; riboflavine, 6 mg; pyridoxine-HCL, 6 mg; nicotinic acid, 30 mg; Ca pantothenate, 15 mg; folic acid, 2 mg; biotin, 0.2 mg; cyanocobalamine, 0.025 mg; 1 g choline chloride.
[3] Ferrous sulfate hydrate contained 30 % iron.

**TABLE 2**
*Fatty acid composition and α-tocopherol concentrations of the dietary fats*

|                                        | Lard | Salmon oil |
|----------------------------------------|------|------------|
| Fatty acids[1]                         | *g/100 g fatty acids* | |
| 14:0                                   | 1.6  | 5.9        |
| 16:0                                   | 26.9 | 13.2       |
| 16:1 (n-7)                             | 1.9  | 7.4        |
| 18:0                                   | 18.4 | 2.6        |
| 18:1 (n-9)                             | 36.3 | 13.2       |
| 18:2 (n-6)                             | 9.4  | 2.2        |
| 18:3 (n-3)                             | 0.9  | 0.8        |
| 20:1 (n-9)                             | 0.8  | 7.9        |
| 20:4 (n-6)                             | -    | 1.6        |
| 20:5 (n-3)                             | -    | 9.5        |
| 22:5 (n-3)                             | -    | 0.8        |
| 22:6 (n-3)                             | -    | 8.8        |
| Tocopherols                            | *mg/kg* | |
| α-tocopherol                           | 18   | 83         |
| All-rac-α-tocopheryl acetate (supplemented) | 569 | 476   |
| Total tocopherol equivalents[2]        | 400  | 400        |

[1] Fatty acids in quantities <0.5 g/100g are not reported.
[2] Total tocopherol equivalents were calculated according to biological activities assessed in rat assays (16,17): mg total tocopherol equivalents = mg α-tocopherol + (mg all-*rac*-α-tocopheryl acetate X 0.67).

The diets were prepared weekly, freeze-dried and stored at 4°C. Lipid peroxidation was monitored by measuring the peroxide value. The peroxide value, determined in diets that were 8 d old, was 1.8 mEq $O_2$/kg diet in the salmon oil diets and 0.95 mEq $O_2$/kg diet in the lard diets.

Diets were administered in restricted amounts to standardize the feed intake. Feeding took place once daily at 0800 h. The amount of food offered daily was 18 g. Water was freely available from nipple drinkers. The diets were fed for 12 wk.

*Sample preparation.* Feces of five rats per group were collected from wk 8 to 12 to determine the digestibility of the iron. At the end of the feeding period, the rats were deprived of food overnight, anesthetized with diethyl ether and killed by decapitation. Blood was collected into heparinized polyethylene tubes and EDTA-treated tubes. Plasma was prepared by centrifuging the heparinized blood (1100 X g, 10 min), and the remaining red blood cells (RBC) were washed three times with 9 g/L sodium chloride solution. Liver and duodenum were removed. Identical sections of the duodenum were cut open lengthwise and mucosa was scraped from duodenal tissue with a clean microscope slide. All samples were immediately shock-frozen in liquid nitrogen and stored at -20°C pending analysis.

*Hematological parameters.* Whole EDTA blood was used immediately for hematocrit and hemoglobin determination as well as counting of different cell type numbers using "Buffy Coat" profiles with the VetAutoRead hematology system (Becton Dickinson, Wörrstadt, Germany). After uptake of the fluorescent dye acridine orange, different cell types were identified by their different fluorescent properties.

*Iron analysis.* Atomic absorption spectrometry (model # 3300, Perkin Elmer, Rodgau-Jügesheim, Germany) was used to determine the iron concentrations in diets, liver and feces. Aliquots of freeze-dried liver were dissolved in nitric acid and ashed under pressure at 170°C for 6 h. The ashes were dissolved in deionized water. Aliquots of diet and dried fecal samples were ashed at 550°C for 16h. The ashes were dissolved in hydrochloric acid at boiling temperature. All samples were filtered, measured at 248.3 nm and calculated with reference to standards.

*Antioxidant status.* Concentrations of α-tocopherol in plasma, liver, and dietary fats were determined by HPLC (HP 1100, Hewlett Packard, Waldbronn, Germany) (18). Samples (200 μL plasma, 50 mg liver) were mixed with 1 mL of 0.1 g/L pyrogallol solution (ethanol, absolute) and 150 μL of saturated sodium hydroxide solution. This mixture was heated for 30 min at 70°C, and tocopherols were extracted with n-hexane. Dietary fat samples were diluted 1:100 with n-hexane. Individual tocopherols of the extracts were separated isocratically using a mixture of n-hexane and 1,4 dioxane (96:4, v/v) as the mobile phase and a LiChrosorb Si-60 column (5-μm particle size, 250 mm length, 4 mm i.d., Merck, Darmstadt, Germany), and detected by fluorescence (excitation wavelength, 295 nm; emission wavelength, 330 nm).

Catalase activity was determined in liver homogenate at 25°C using hydrogen peroxide as the substrate by the method of Beers and Sizer (19). One unit of catalase activity is defined as the amount consuming 1 μmol hydrogen peroxide/min. Total superoxide dismutase (SOD) activities of duodenal mucosa homogenate and RBC were determined using the method of Marklund and Marklund (20) with pyrogallol as the substrate. One unit of SOD activity is defined as the amount of enzyme required to inhibit the autoxidation of pyrogallol by 50%. Glutathione peroxidase (GSH-Px) activity in plasma was measured with t-butyl hydroperoxide at 25°C according to the method of Paglia and Valentine (21) as modified by Levander et al. (22). One unit of GSH-Px activity is defined as 1 μmol NADPH oxidized/min. Total glutathione (GSH) concentration was determined in protein-free liver homogenates according to Griffith (23) with glutathione reductase and Ellman´s reagent. Calibration was performed using a standard curve. Oxidized glutathione (GSSG) was detected after derivatization of GSH with 2-vinylpyridine. The concentration of reduced GSH was calculated as total GSH – 2 X GSSG. Sample protein content was determined according to the

method of Lowry et al. (24). Enzyme activities of RBC, liver, and mucosa were expressed per milligram or gram protein.

*Lipid analysis.* The fatty acid composition of the dietary fats was determined by gas chromatography. Fats were methylated with trimethylsulfonium hydroxide (25). Fatty acid methyl esters (FAME) were separated by gas chromatography, using a system (HP 5890, Hewlett Packard GmbH, Böblingen, Germany) equipped with an automatic on-column injector, a polar capillary column (30 m FFAP, 0.53 mm i.d., Macherey and Nagel, Düren, Germany) and a flame ionisation detector. Helium was used as the carrier gas with a flow rate of 5.4 mL/min. FAME were identified by comparing their retention times with those of individually purified standards. Concentrations of total cholesterol and triglycerides in plasma were determined using commercially available enzymatic reagent kits (Merck; Cat. Nos. 1.14830, 1.14856).

*Thiobarbituric acid reactive substances.* TBARS were measured in plasma, liver, and mucosal homogenates using a modified version of the TBARS assay (26). Sample aliquots were mixed with thiobarbituric acid reagent (8 g/L thiobarbituric acid with 7% perchloric acid, 2:1,v/v) and heated for 60 min at 95°C. TBARS were extracted with n-butanol, and absorption was measured at 532 nm. Concentrations were calculated via a standard curve with 1,1,3,3,-tetraethoxypropan.

*Cholesterol oxidation products.* COP were determined in liver by gas chromatography/mass spectrometry in selected ion monitoring mode according to Mori et al. (27) with modifications. Total lipids of liver were extracted with a hexane/isopropanol mixture (3:2, v/v) (28). Aliquots of the extracts were mixed with 20 μg of the internal standard 5α-cholestane. The probes were dried under nitrogen, mixed with 2 mL of 1 mol/L methanolic potassium hydroxide, and incubated for 18 h at room temperature. Cholesterol oxides were extracted with 2 mL diethyl ether into the nonsaponifiable fraction, separated, dried under nitrogen, and dissolved in 100 μL pyridine. After derivatisation of the oxides with 100 μL 0.1 g/L bis(trimethylsilyl)-trifluoroacetamide, samples were separated using a nonpolar capillary column (DB-5, 30 m X 0.25 mm i.d., J&W Scientific, Folsom, CA) and detected by single ion monitoring. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The cholesterol oxides 7β-hydroxycholesterol, 7-ketocholesterol, cholesterol-5α,6α-epoxide, cholesterol-5β,6β-epoxide, cholestanetriol, and 25-hydroxycholesterol were identified by comparing their retention times with those of authentic standards and quantified with the internal standard.

*Statistics.* Treatment effects were analyzed by two-way ANOVA using the Minitab statistical software (Release 13, Minitab, State College, PA). Classification factors were iron level and fat type, as well as their interaction. Means of the four treatment groups were compared by Fisher`s multiple range test. Differences were considered significant if $P<0.05$. Values in the text are means ± SD.

## RESULTS

*Body weight.* Body weight gains during the experimental period were slightly, but significantly higher in the rats fed the high iron diets than in the rats fed the iron-adequate diets (n = 20; adequate iron diets, 212 ± 20.4 g; high iron diets, 227 ± 16.8 g; $P<0.05$). Rats fed salmon oil diets had higher body weight gains than rats fed lard diets (n = 20; salmon oil diets, 231 ± 16.1 g; lard diets, 208 ± 17.4 g; $P<0.05$).

*Iron status.* Liver iron concentration was significantly influenced by dietary iron concentration and dietary fat (**Table 3**). It was significantly higher in rats fed high iron diets than in rats fed adequate iron diets. The difference between rats fed the high iron diets and rats fed the adequate iron diets was ~20%. Rats fed the lard diet had significantly higher hepatic iron concentrations than rats fed the salmon oil diet. Fecal iron concentration, iron excretion, and apparent iron digestibility were significantly affected by the dietary iron

concentration, but not by the dietary fat type. The iron concentration of the feces and the amount of iron excreted with the feces were approximately four times higher in rats fed the high iron diets than in rats fed the adequate iron diets. In the rats fed the adequate iron diets, ~25% of the iron was apparently absorbed; in the rats fed the high iron diets, the apparent digestibility of iron was slightly negative, indicating that in a given period, they excreted more iron than they consumed.

**TABLE 3**

*Iron concentration in liver and feces and apparent digestibility of iron of rats fed diets with two levels of iron (50 or 500 mg/kg supplemented) containing either lard or salmon oil at 100 g/kg diet for 12 wk* [1,2,3]

| Fat | | Lard | | Salmon oil | | ANOVA (P<) | | |
|---|---|---|---|---|---|---|---|---|
| Iron supplemented, *mg/kg diet* | n | 50 | 500 | 50 | 500 | Iron | Fat | Iron x Fat |
| Iron concentration | | | | | | | | |
| Liver, *μmol/g DM* | 10 | $8.04 \pm 1.41^b$ | $9.68 \pm 1.85^a$ | $6.63 \pm 1.48^b$ | $7.89 \pm 1.46^b$ | 0.01 | 0.01 | NS |
| Feces, *μmol/g DM* | | | | | | | | |
| wk 8 | 5 | $23.4 \pm 2.8^b$ | $115 \pm 8^a$ | $23.5 \pm 2.1^b$ | $114 \pm 8^a$ | 0.001 | NS | NS |
| wk 12 | 5 | $24.4 \pm 2.9^b$ | $113 \pm 5^a$ | $23.7 \pm 2.7^b$ | $111 \pm 13^a$ | 0.001 | NS | NS |
| Fecal excretion of iron, *mmol/7d* | | | | | | | | |
| wk 8 | 5 | $0.24 \pm 0.01^b$ | $1.25 \pm 0.08^a$ | $0.25 \pm 0.04^b$ | $1.17 \pm 0.07^a$ | 0.001 | NS | NS |
| wk 12 | 5 | $0.27 \pm 0.01^b$ | $1.30 \pm 0.09^a$ | $0.27 \pm 0.01^b$ | $1.22 \pm 0.17^a$ | 0.001 | NS | NS |
| Apparent digestibility of iron, *%* | | | | | | | | |
| wk 8 | 5 | $26.5 \pm 3.7^a$ | $-5.70 \pm 2.13^b$ | $28.4 \pm 5.1^a$ | $-1.39 \pm 6.09^b$ | 0.001 | NS | NS |
| wk 12 | 5 | $22.1 \pm 2.9^a$ | $-4.64 \pm 7.61^b$ | $24.4 \pm 3.6^a$ | $1.99 \pm 1.37^b$ | 0.001 | NS | NS |

[1] Results are means ± SD.
[2] Means in a row without a common superscript letter differ, P<0.05.
[3] Abbreviations: DM, dry matter; NS, non significant (P≥0.05).

***Hematological variables.*** Hematological variables did not differ among the four groups (overall means ± SD, n = 40; hematocrit, 0.44 ± 0.05; hemoglobin, 14.5 ± 1.93 g/L; reticulocytes, 2.84 ± 1.08%; leukocyte count, 7.46 ± 3.71 X $10^9$/L; granulocyte count, 5.18 ± 2.88 X $10^9$/L). The numbers of lymphocytes and monocytes, however, were higher in the rats fed salmon oil (2.82 ± 1.49 X $10^9$/L) than in the rats fed lard (1.73 ± 0.58 X $10^9$/L, P<0.05).

***Antioxidant status.*** The concentrations of TBARS in plasma, liver, and mucosa were not influenced by the dietary iron concentration (**Table 4**), but the dietary fat affected the concentrations of TBARS in plasma and liver. Rats fed salmon oil diets had significantly higher concentrations of TBARS in plasma and liver than rats fed lard diets. The concentrations of TBARS in the mucosa did not differ among the four treatment groups.

**TABLE 4**

*Concentration of thiobarbituric acid reactive substances in plasma, liver and mucosa of rats fed diets with two levels of iron (50 or 500 mg/kg supplemented) containing either lard or salmon oil at 100 g/kg diet for 12 wk* [1,2,3]

| Fat | Lard | | Salmon oil | | ANOVA (P<) | | |
|---|---|---|---|---|---|---|---|
| Iron supplemented, *mg/kg diet* | 50 | 500 | 50 | 500 | Iron | Fat | Iron x Fat |
| Plasma, *μmol/l* | $14.0 \pm 1.4^b$ | $16.0 \pm 2.5^b$ | $29.6 \pm 7.7^a$ | $29.6 \pm 8.5^a$ | NS | 0.001 | NS |
| Liver, *nmol/g wet tissue* | $38.0 \pm 1.9^b$ | $42.1 \pm 2.0^b$ | $62.4 \pm 17.9^a$ | $64.8 \pm 3.6^a$ | NS | 0.001 | NS |
| Mucosa, *nmol/g protein* | $92.8 \pm 30.9$ | $92.2 \pm 34.6$ | $95.8 \pm 37.2$ | $126 \pm 30$ | NS | NS | NS |

[1] Results are means ± SD, n=10.
[2] Means without a common superscript letter differ, P<0.05.
[3] Abbreviations: NS, non significant (P≥0.05).

The concentrations of hepatic COP were affected by the dietary iron concentration and fat **(Table 5)**. The concentrations of all COP measured were one- to threefold higher in rats fed salmon oil diets than in rats fed lard diets. The effect of the dietary iron on the concentrations of some COP depended on the type of dietary fat. Rats fed the salmon oil diet with high iron concentration had significantly higher concentrations of 7β-hydroxycholesterol, 7-ketocholesterol, and cholestanetriol in the liver than rats fed the salmon oil diet with adequate iron. In contrast, in rats fed the lard diet with high iron concentration, only the concentration of cholestanetriol was significantly higher than in rats fed the lard diet with adequate iron; the concentrations of 7β-hydroxycholesterol and 7-ketocholesterol did not differ between these two groups. The concentrations of cholesterol epoxides were not affected by the dietary iron concentration. There was a significant interaction between iron and fat on the concentrations of 7β-hydroxycholesterol and 7-ketocholesterol.

**TABLE 5**

*Concentrations of various cholesterol oxidation products in liver of rats fed diets with two levels of iron (50 or 500 mg/kg supplemented) containing either lard or salmon oil at 100 g/kg diet for 12 wk*[1,2,3]

| Fat | Lard | | Salmon oil | | ANOVA (P<) | | |
|---|---|---|---|---|---|---|---|
| Iron supplemented, *mg/kg diet* | 50 | 500 | 50 | 500 | Iron | Fat | Iron x Fat |
| *nmol/g wet liver* | | | | | | | |
| 7β-Hydroxycholesterol | $9.81 \pm 2.87^c$ | $11.1 \pm 2.4^c$ | $22.2 \pm 5.2^b$ | $42.8 \pm 15.6^a$ | 0.001 | 0.001 | 0.01 |
| 7-Ketocholesterol | $7.99 \pm 4.69^b$ | $7.39 \pm 2.78^b$ | $12.6 \pm 3.5^b$ | $26.8 \pm 10.7^a$ | 0.01 | 0.001 | 0.01 |
| Cholesterol 5α,6α-epoxide | $5.75 \pm 1.59^b$ | $5.79 \pm 0.40^b$ | $14.3 \pm 6.5^a$ | $14.1 \pm 4.2^a$ | NS | 0.001 | NS |
| Cholesterol 5β,6β-epoxide | $8.78 \pm 3.38^b$ | $8.07 \pm 2.56^b$ | $18.8 \pm 9.9^a$ | $17.5 \pm 4.9^a$ | NS | 0.001 | NS |
| Cholestanetriol | $0.59 \pm 0.19^c$ | $1.24 \pm 0.24^b$ | $1.47 \pm 0.36^b$ | $1.97 \pm 0.38^a$ | 0.001 | 0.001 | NS |
| 25-Hydroxycholesterol | $0.17 \pm 0.05^b$ | $0.24 \pm 0.05^b$ | $0.30 \pm 0.10^{ab}$ | $0.37 \pm 0.10^a$ | 0.05 | 0.001 | NS |

[1] Results are means ± SD, n=10.
[2] Means without a common superscript differ, P<0.05.
[3] Abbreviations: NS, non significant (P≥0.05).

Of the antioxidative enzymes measured, only the activity of hepatic catalase was affected by the dietary iron concentration **(Fig. 1)**. Rats fed high iron diets had significantly greater activity than rats fed adequate iron diets. Dietary fat had no effect on hepatic catalase activity. The activities of GSH-Px in plasma as well as of SOD in RBC and mucosa were not affected by either dietary iron or dietary fat (overall means ± SD, n = 40; GSH-Px, 2.51 ± 1.01 kU/L; RBC SOD, 9.97 ± 2.13 U/mg protein; mucosa SOD, 7.62 ± 2.23 U/mg protein).

The concentration of hepatic glutathione was influenced by the dietary iron and fat **(Fig. 2)**. Rats fed the high iron diets had significantly greater concentrations of total GSH, reduced GSH, and GSSG in the liver than rats fed adequate iron diets. Rats fed salmon oil diets had significantly higher concentrations of total GSH as well as GSSG than rats fed lard diets; the concentration of reduced GSH was not affected by dietary fat.

The concentrations of α-tocopherol in plasma and liver were not influenced by the dietary iron. However, rats fed the lard diets had higher concentrations of α-tocopherol in liver and plasma than rats fed the salmon oil diets (means ± SD, n = 20; liver, 1.71 ± 0.54 vs. 0.71 ± 0.21 μmol/mmol triglycerides plus cholesterol, P<0.001; plasma, 6.73 ± 1.64 vs. 5.08 ± 1.56 μmol/mmol triglycerides plus cholesterol, P<0.05).
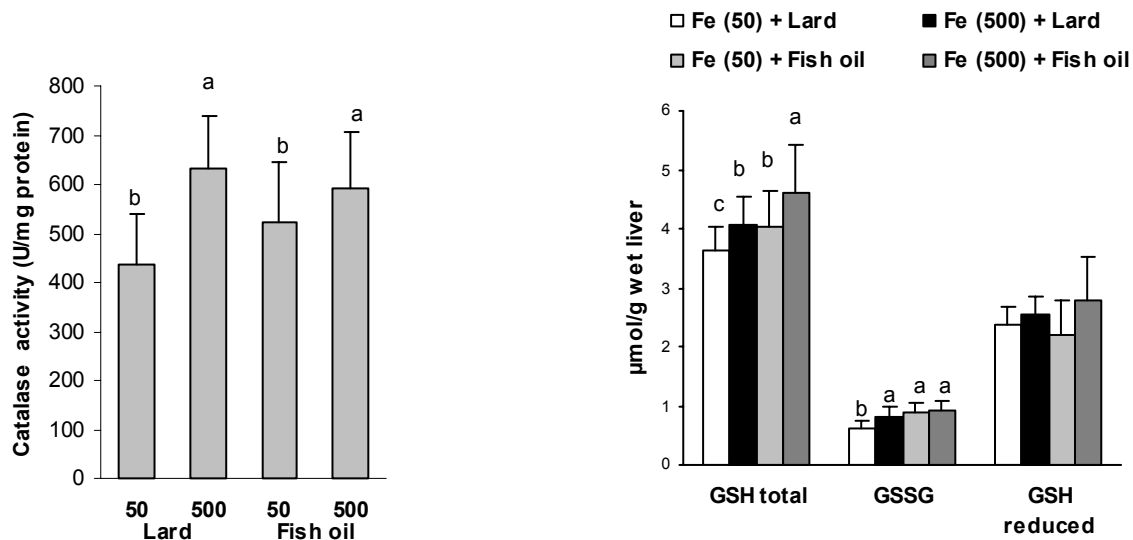




**FIGURE 1** Hepatic catalase activity in rats fed diets with two levels of iron (50 or 500 mg/kg supplemented) containing either lard or salmon oil at 100 g/kg diet for 12 wk. Values are means ± SD, n=10. Means with different letters differ by Fisher´s multiple range test, P<0.05. Results of ANOVA: iron, P<0.01; fat, non significant; iron x fat, non significant.

**FIGURE 2** Concentrations of total glutathione (GSH total), oxidized glutathione (GSSG) and reduced glutathione (GSH reduced) in the liver of rats fed diets with two levels of iron (50 or 500 mg/kg supplemented) containing either lard or salmon oil at 100 g/kg diet for 12 wk. Values are means ± SD, n=10. Means with different letters differ, P<0.05. Results of ANOVA: GSH total: iron, P<0.01; fat, P<0.05; iron x fat, non significant. GSSG: iron, P<0.05; fat, P<0.001; iron x fat, non significant. GSH reduced: iron, P<0.05; fat, non significant; iron x fat, non significant.

## DISCUSSION

We investigate the effect of high dietary iron concentrations on the antioxidant status of rats fed PUFA-rich fat compared with rats fed a fat low in PUFA. In addition to conventional components of the antioxidant system, we measured hepatic COP. To our knowledge, the effect of high iron diets on the formation of COP in association with various dietary fats has not previously been investigated.

Surprisingly, feeding the high iron diets (500 mg iron supplemented/kg) increased the hepatic iron concentration only 20% compared with feeding the iron-adequate diets (50 mg iron supplemented/kg). The hepatic iron concentrations in rats fed the high iron diets were lower than those observed in similar studies in which rats were fed high iron diets (9,10). The difference between those studies and our own results could be attributable to the age of the rats used; in our study, adult rats were used, whereas Bristow-Craig et al. (10) and Soyars and Fisher (9) studied growing rats. The moderate accumulation of iron in the livers of rats fed the high iron diets could also be attributed to a marked reduction in the apparent digestibility of

iron induced by a high iron intake. A net loss of iron was observed in the rats fed the high iron diets, suggesting a mechanism for preventing iron overload via increased excretion. The apparent digestibility of iron depends on the body's iron stores (1). In a study by Reddy and Cook (29), feeding a diet with 500 mg iron/kg lowered the rate of iron digestibility from 50 to 27% within only 1 wk; in a study by Rimbach and Pallauf (30) increasing the dietary iron concentration from 30 to 300 mg/kg reduced the apparent digestibility of iron from 25 to 8%. The observation that rats fed salmon oil have lower concentrations of hepatic iron than rats fed lard is consistent with other published studies that demonstrated that fish oil reduces the absorption of iron (31,32). The amounts of iron excreted with the feces in the wk 8 and 12 of the experiment, however, were not different between rats fed lard and those fed salmon oil. The differences in the absorption of iron may have occurred during the earlier phase of the experiment as a result of the dietary fat used, which would explain the different hepatic iron concentrations.

As expected, feeding the salmon oil diets caused a marked oxidative stress compared with feeding the lard diets. This was shown by greater concentrations of TBARS in liver and plasma, reduced concentrations of α-tocopherol in liver and plasma and greater concentrations of total and oxidized glutathione in the liver. This finding agrees with many other studies that also reported oxidative stress in animals fed diets containing marine oils (12,33). The present study, moreover, showed for the first time that dietary salmon oil also enhances the formation of COP in the liver. Cholesterol is susceptible to oxidation under a variety of conditions (13). In contact with air, cholesterol may autoxidize via various active oxygen species, whereas in cell membranes, cholesterol can be oxidized either enzymatically or via free radical-mediated peroxidation processes (13,34). We assume that the oxidation of cholesterol was initiated by the autoxidation of highly unsaturated fatty acids, which are very susceptible to oxidation. Feeding fish oil enriches cell membranes in highly unsaturated fatty acids such as eicosapentaenoic acid or docosahexaenoic acid (35).

The high iron diet did not dramatically stress the antioxidant system, irrespective of the dietary fat as shown by minor or no changes in TBARS, GSH-Px, SOD, GSH or α-tocopherol. This agrees with some other studies performed with rats that also did not find effects of a moderate dietary iron excess (≤400 mg iron/kg diet) on activities of antioxidant enzymes or concentrations of α-tocopherol, glutathione, and malondialdehyde in the liver (10,12). In contrast to the other antioxidant enzymes measured, the activity of catalase was increased by feeding high iron diets. This may have been because of increased iron concentrations in the liver rather than to induction by oxidative stress because the activity of catalase depends on iron status.

The finding that the concentrations of TBARS in intestinal mucosa were not elevated by feeding high iron diets suggests that the high iron concentrations in the chyme did not enhance lipid peroxidation in the intestine. Some other studies (36,37) suggest that high iron concentrations in the intestine enhance the formation of lipid peroxides in intestinal mucosa.

The present study showed for the first time that dietary iron excess could enhance the formation of COP in the liver, particularly in rats fed a diet rich in PUFA. The fact that the effect of high iron diets on the formation of COP was stronger in rats fed salmon oil than in rats fed lard also suggests that the formation of COP is a consequence of oxidation of PUFA. Interestingly, increased concentrations of 7-hydroxycholesterol and 7-ketocholesterol were also found in brains and visceral organs of aceruloplasminemic patients (14) who have extremely high concentrations of iron in brain and visceral organs, which are strongly prooxidative and enhance the formation of lipid peroxidation products. Our study shows that even a modest increase in dietary iron enhances the formation of COP in the liver when the antioxidant system is stressed by other factors, such as dietary highly unsaturated fatty acids.

Overall, the results of our research indicate that diets with high concentrations of highly unsaturated fatty acids stimulate the formation of COP in the liver and that simultaneous

dietary iron excess amplifies this effect. This is critical given that several unfavorable effects of COP have been described. They are associated with mutagenic and carcinogenic events (38-40) and are involved in the pathogenesis of atherosclerosis (41,42). COP are transported as constituents of lipoproteins (43) and may therefore leave the liver. Once they have entered the bloodstream, they could exert their toxic effects on various cell types, as described in *in vitro* studies of endothelial cells (44) and smooth muscle cells (45). Studies investigating the effects of PUFA and of high dietary iron concentrations should therefore include an investigation of hepatic cholesterol oxides.

# LITERATURE CITED

1. Yip, R. & Dallman, P. R. (1996) Iron. In: Present Knowledge in Nutrition (Ziegler, E. E. & Filer, L. J. Jr., eds.), pp. 277-292. ILSI Press, Washington, DC.

2. Sheth, S., Brittenham, G. M. (2000) Genetic disorders affecting proteins of iron metabolism: clinical implications. Annu. Rev. Med. 51: 443-464.

3. Halliwell, B. & Gutteridge, J. M. (1986) Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. Arch. Biochem. Biophys. 246: 501-514.

4. Toyokuni, S. (1996) Iron-induced carcinogenesis: the role of redox regulation. Free Radic. Biol. Med. 20: 553-566.

5. de Valk, B. & Marx, J. J. (1999) Iron, atherosclerosis, and ischemic heart disease. Arch. Intern. Med. 159:1542-1548.

6. Ascherio, A., Willett, W. C., Rimm, E. B., Giovannucci, E. L. & Stampfer, M. J. (1994) Dietary iron intake and risk of coronary disease among men. Circulation 89: 969-974.

7. Nelson, R. L. (1992) Dietary iron and colorectal cancer risk. Free Radical Biol. Med. 12: 161-168.

8. Wurzelmann, J. I., Silver, A., Schreinemachers, D. M., Sandler, R. S. & Everson, R. B. (1996) Iron intake and the risk of colorectal cancer. Cancer Epidemiol. Biomarkers Prev. 5: 503-507.

9. Soyars, K. E. & Fischer, J. G. (1998) Iron Supplementation Does Not Affect Cell Proliferation or Aberrant Crypt Foci Development in the Colon of Sprague-Dawley Rats. J. Nutr. 128: 764-770.

10. Bristow-Craig, H. E., Strain, J. J. & Welch, R. W. (1994) Iron Status, Blood Lipids and Endogenous Antioxidants in Response to Dietary Iron Levels in Male and Female Rats. Internat. J. Vit. Nutr. Res. 64: 324-329.

11. Dabbagh, A. J., Mannion, T., Lynch, S. M. & Frei, B. (1994) The Effect of Iron Overload on Rat Plasma and Liver Oxidant Status in vivo. Biochem. J. 300: 799-803.

12. Ibrahim, W., Lee, U. S., Yeh, C. C., Szabo, J., Bruckner, G. & Chow, C. K. (1997) Oxidative stress and antioxidant status in mouse liver: effects of dietary lipid, vitamin E and iron. J. Nutr. 127: 1401-1406.

13. Peng, S.-K. & Morin, R.-J., eds. (1992) Biological effects of cholesterol oxides. CRC Press, London.

14. Miyajima, H., Adachi, J., Kohno, S., Takahashi, Y., Ueno, Y. & Naito, T. (2001) Increased oxysterols associated with iron accumulation in the brains and visceral organs of acaeruloplasminaemia patients. QJM. 94: 417-422.

15. Yoshida, H. & Kajimoto, G. (1989) Effect of dietary vitamin E on the toxicity of autoxidized oil to rats. Ann. Nutr. Metab. 33: 153-161.

16. Horwitt, M. K. (1976) Vitamin E: A Reexamination. Am. J. Clin. Nutr. 29: 569-578.

17. Weiser, H. & Vecchi, M. (1982) Stereoisomers of Alpha-Tocopheryl Acetate. II. Biopotencies of All Eight Stereoisomers, Individually or in Mixtures, as Determined by Rat Resorption-Gestation Tests. Internat. J. Vit. Nutr. Res. 52: 351-370.

18. Balz, M. K., Schulte, E. & Thier, H. P. (1993) Simultanous determination of $\alpha$-tocopherolacetate, tocopherols and tocotriens by HPLC with fluorescence detection in foods. Fat Sci. Technol. 95: 215-220.

19. Beer, R. F. & Sizer, I. W. (1952) A spectrophotometric method for measuring the breakdown of hydrogen by catalase. J. Biol. Chem. 195: 133-140.

20. Marklund, S. L. & Marklund, G. (1974) Involvement of superoxide anion radical in the autoxidation of pyrogallol and a convenient assay of superoxide dismutase. Eur. J. Biochem. 47: 469-474.

21. Paglia, D. E. & Valentine, W. N. (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70: 158-169.

22. Levander, O. A., DeLoach, D. P., Morris, V. C. & Moser, P. B. (1983) Platelet glutathione peroxidase activity as an index of selenium status in rats. J. Nutr. 113: 55-63.

23. Griffith, O. W. (1986) Glutathione and glutathione disulfide. In: Methods of Enzymatic Analysis. Bergmeyer, H. U. (ed.) 3. Edition, VHC Verlagsgesellschaft mbH, Weinheim, pp. 521-529.

24. Lowry, O. H., Rosebrough N. J., Farr, A. L. & Randall, R. J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 293: 265-275.

25. Butte, W. (1983) Rapid method for the determination of fatty acid profiles from fats and oils using trimethylsulfonium hydroxide for transesterification. J. Chromatogr. 261: 142-145.

26. Halliwell, B. & Gutteridge, J. M. C. (eds.) (1989) Free radicals in Biology and Medcine. Clarendon Press, Oxford.

27. Mori, T. A., Croft, K. D., Puddey, I. B. & Beilin, L. J. (1996) Analysis of native and oxidized low-density lipoprotein oxysterols using gas chromatography-mass spectrometry with selective ion monitoring. Redox Report 2: 25-34.

28. Hara, A. & Radin, S. N. (1978) Lipid extraction of tissues with a low-toxicity solvent. Anal. Biochem. 90: 420-426.

29. Reddy, M. B. & Cook, J. D. (1991) Assessment of dietary determinants of nonheme-iron absorption in humans and rats. Am. J. Clin. Nutr. 54: 723-728.

30. Rimbach, G. & Pallauf, J. (1998) Phytic acid inhibits free radical formation in vitro but does not affect liver oxidant or antioxidant status in growing rats. J. Nutr. 128: 1950-1955.

31. Araujo, J. A., Romano, E. L., Brito, B. E., Parthe, V., Romano, M., Bracho, M., Montano, R. F. & Cardier, J. (1995) Iron Overload Augments the Development of Atherosclerotic Lesions in Rabbits. Arterioscler. Thromb. Vasc. Biol.15: 1172-1180.

32. Sullivan, J. L. (1989) The iron paradigm of ischemic heart disease. Am. Heart J. 117: 1177-1188.

33. Mouri, K., Ikesu, H., Esaka, T., Igarashi, O. (1984) The influence of marine oil intake upon levels of lipids, alpha-tocopherol and lipid peroxidation in serum and liver of rats. J. Nutr. Sci. Vitaminol. 30: 307-318.

34. Schroepfer, G. J. (2000) Oxysterols: modulators of cholesterol metabolism and other processes. Physiol. Rev. 80: 361-554.

35. Kaasgaard, S. G., Holmer, G., Hoy, C. E., Behrens, W. A., Beare-Rogers, J. L. (1992) Effects of dietary linseed oil and marine oil on lipid peroxidation in monkey liver in vivo and in vitro. Lipids 27: 740-745.

36. Lund, E. K., Fairweather-Tait, S. J., Wharf, S. G. & Johnson, I. T. (2001) Chronic exposure to high levels of dietary iron fortification increases lipid peroxidation in the mucosa of the rat large intestine. J. Nutr. 131: 2928-2931.

37. Rimbach, G., Markant, A., Most, E. & Pallauf, J. (1997) Liver and colon oxidant status in growing rats fed increasing levels of dietary iron. J. Trace Elem. Med. Biol. 11: 99-104.

38. Morin, R. J., Hu, B., Peng, S. K. & Sevanian, A. (1991) Cholesterol oxides and carcinogenesis. J. Clin. Lab. Anal. 5: 219-225.

39. Sevanian, A. & Peterson, A. R. (1986) The cytotoxic and mutagenic properties of cholesterol oxidation products. Food Chem. Toxicol. 24: 1103-1110.

40. Gruenke, L. D., Wrensch, M. R., Petrakis, N. L., Miike, R., Ernster, V. L. & Craig, J. C. (1987) Breast fluid cholesterol and cholesterol epoxides: relationship to breast cancer risk factors and other characteristics. Cancer Res. 47: 5483-5487.

41. Brown, A. J. & Jessup, W. (1999) Oxysterols and atherosclerosis. Atherosclerosis 142: 1-28.

42. Peng, S. K., Hu, B. & Morin, R. J. (1991) Angiotoxicity and atherogenicity of cholesterol oxides. J. Clin. Lab. Anal. 5: 144-152.

43. Peng, S. K., Phillips, G. A., Xia, G. Z. & Morin, R. J. (1987) Transport of cholesterol autoxidation products in rabbit lipoproteins. Atherosclerosis 64: 1-6.

44. Sevanian, A., Berliner, J. & Peterson, H. (1991) Uptake, metabolism, and cytotoxicity of isomeric cholesterol-5,6-epoxides in rabbit aortic endothelial cells. J. Lipid Res. 32: 147-155.

45. Hughes, H., Mathews, B., Lenz, M. L. & Guyton, J. R. (1994) Cytotoxicity of oxidized LDL to porcine aortic smooth muscle cells is associated with the oxysterols 7-ketocholesterol and 7-hydroxycholesterol. Arterioscler. Thromb. 14: 1177-1185.

**3.2    Effekt von Fischöl und oxidiertem Cholesterin auf die 7β-Hydroxycholesterol-Konzentrationen in der Ratte bei unterschiedlicher Vitamin E-Versorgung**

***ABSTRACT***   *Two experiments with rats were carried out to investigate the effect of dietary fish oil and oxidized cholesterol on the concentration of 7β-hydroxycholesterol, an oxysterol of mainly nonenzymatic origin, in liver, plasma, low density lipoproteins and erythrocytes of rats at different vitamin E supply. In Experiment 1, four groups of rats received diets with coconut oil or salmon oil (100 g/kg diet) and vitamin E concentrations of 40 or 240 mg α-tocopherol equivalents/kg. In Experiment 2, eight groups of rats received diets supplemented with pure or oxidized cholesterol (5 g/kg), coconut oil or salmon oil (100 g/kg diet) and vitamin E concentrations of 40 or 240 mg α-tocopherol equivalents/kg. Rats fed the salmon oil diets had significantly higher concentrations of 7β-hydroxycholesterol in liver, plasma, low density lipoproteins and erythrocytes than rats fed coconut oil diets. Rats fed the diets supplemented with oxidized cholesterol had significantly higher concentrations of 7β-hydroxycholesterol in all the samples analysed than rats fed pure cholesterol. Increasing the dietary vitamin E concentration from 40 to 240 mg α-tocopherol equivalents/kg diet reduced the concentration of 7β-hydroxycholesterol in plasma; the concentrations of 7β-hydroxycholesterol in liver, low density lipoproteins and erythrocytes were not influenced by the dietary vitamin E concentration. The study suggests that diets with fish oil or oxidized cholesterol are critical with respect to the formation of 7β-hydroxycholesterol.*

**Keywords:** 7β-hydroxycholesterol, fish oil, oxidized cholesterol, vitamin E, oxysterols, rat.

## 1 Introduction

As compared to 7α-hydroxycholesterol, 27-hydroxycholesterol, 24S-hydroxycholesterol and 4β-hydroxycholesterol, which are the dominating oxysterols in human plasma, 7β-hydroxycholesterol (7β-OH-Chol) is a minor oxysterol in the human circulation. However, 7β-OH-Chol is of scientific interest due to its potential involvement in the aetiology and progression of atherosclerosis [1, 2]. It has been shown that 7β-OH-Chol exerts cytotoxic effects towards the cells of the vasculature [3] and it appears that increased levels of 7β-OH-Chol are linked to a higher risk of atherosclerosis [4, 5]. In contrast to the aforementioned oxysterols, which are formed enzymatically through oxygenation by different cytochrome P-450 species, most of the 7β-OH-Chol in the circulation is formed nonenzymatically in the presence of reactive oxygen species (ROS) [6, 7]. Therefore, the amount of 7β-OH-Chol in the organism is the result of endogenous formation via peroxidation of cholesterol and dietary intake regarding that 7β-OH-Chol is readily absorbed in the intestine from oxysterol-containing foods [8, 9]. Thus, it is of great interest for practical nutrition wether concentrations of 7β-OH-Chol in plasma, lipoproteins and tissues can be influenced by dietary regimes. Therefore, several studies have been carried out to investigate the impact of various nutrients on the concentrations of 7β-OH-Chol in plasma and tissues [10-12].

The endogenous formation of 7β-OH-Chol is affected in particular by the concentration of cholesterol in membranes and lipoproteins. It was shown that feeding cholesterol-rich diets caused the concentrations of 7β-OH-Chol in plasma to increase markedly [12, 13]. As 7β-OH-Chol is formed in the presence of ROS its endogenous

formation in animal tissues is probably also influenced by the antioxidative status. Some studies suggest that the formation of 7β-OH-Chol from cholesterol in membranes and lipoproteins is promoted by oxidation of polyunsaturated fatty acids (PUFA). ROS formed during oxidation of PUFA can modify cholesterol as a secondary effect [14, 15]. The ingestion of fats containing a high proportion of oxidation-susceptible fatty acids, such as salmon oil, increases the susceptibility of lipids in tissues and lipoproteins to lipid peroxidation [16, 17]. It is therefore reasonable to assume that the endogenous formation of 7β-OH-Chol is also promoted by fats with a high proportion of PUFA. However, less studies exist about the effects of various dietary fats on the concentrations of 7β-OH-Chol in plasma, lipoproteins and tissues. Fat-soluble antioxidants like vitamin E on the other hand inhibit lipid peroxidation [16, 17]. It is therefore reasonable to assume that the endogenous formation of 7β-OH-Chol is suppressed by a high intake of vitamin E. In rabbits fed cholesterol-enriched diets supplementation with vitamins E and C [18] or probucol [6] reduced the concentrations of 7β-OH-Chol in plasma. In humans, dietary vitamin E supplementation reduced plasma 7β-OH-Chol in diabetic patients, but not in healthy controls [19]. Therefore, the effect of antioxidants on the concentration of 7β-OH-Chol in animal tissues requires further studies. Studies on the interaction between the type of dietary fat and vitamin E intake and its effect on the formation of 7β-OH-Chol are however lacking so far. Therefore, the first experiment of this study was conducted to investigate the interaction between dietary fats and vitamin E for the formation of 7β-OH-Chol in different tissues. Salmon oil was used as a source of highly unsaturated, oxidation-susceptible fatty acids. It is often used in practical nutrition for the prevention of coronary heart disease due to its high content of (n-3) PUFA. Coconut oil was used as a control fat with a very low concentration of PUFA.

On the other hand exogenous 7β-OH-Chol from the diet increases the concentration of 7β-OH-Chol in liver and plasma [20, 21]. However, there is less information whether the supply of vitamin E influences the incorporation of 7β-OH-Chol from the diet into lipoproteins and tissues. The second experiment of this study, therefore intended to investigate the interaction between dietary vitamin E and dietary oxidized cholesterol with regards to the concentrations of 7β-OH-Chol in tissues. As we expected that the concentrations of 7β-OH-Chol are influenced by the type of dietary fat we used two different dietary fats, salmon oil and coconut oil. Rats were used as model objects bearing in mind that rats show a different lipoprotein profile and lipoprotein metabolism than humans. However as they are susceptible to the induction of oxidative stress *in vivo* they are widely used to investigate the effect of different feeding regimes on markers of lipid peroxidation such as the formation of 7β-OH-Chol in tissues [10-12]. The concentrations of 7β-OH-Chol in plasma and low density lipoproteins (LDL) were of particular interest in this study with respect to its potential involvement in the development of atherosclerosis. Moreover, the concentrations of 7β-OH-Chol in the liver, the tissue which synthesizes and secretes plasma lipoproteins, and in erythrocytes, to study the effects of dietary treatment on the concentrations of 7β-OH-Chol in a pure membrane fraction, were analysed.

## 2 Materials and methods
### 2.1 Animals
Two experiments were carried out with male Sprague-Dawley rats supplied by Charles River (Sulzfeld, Germany). Experiment 1 included 36 rats with an initial body weight of 72 g (± 9 g, SD) which were assigned to four groups of nine rats each; Experiment 2 included seventy-two rats with an initial body weight of 70 g (± 8 g, SD) which were assigned to eight groups of nine rats each. The animals were kept individually in Macrolon cages in a room maintained at a temperature of 23°C and 50 to 60% relative humidity with lighting from 0600 to 1800 h. All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt.

## 2.2 Diets and feeding

Semisynthetic diets were used. The basal diet used in Experiment 1 consisted of (g/kg diet): corn starch (398), casein (200), saccharose (200), fat (100), cellulose (40), vitamins and minerals (60) and DL-methionine (2). Minerals and vitamins with the exception of vitamin E were supplemented in accordance with the recommendations of the American Institute of Nutrition [22] for rat diets. According to a bifactorial design, the dietary fat (coconut oil vs. salmon oil) and the vitamin E concentration of the diet (40 vs. 240 mg α-tocopherol equivalents per kg) were varied. The basal diet used in Experiment 2 was identical with that used in Experiment 1 with the exception that it was supplemented with 5 g/kg of either pure cholesterol or oxidized cholesterol at the expense of cellulose. According to a three-factorial design, besides the cholesterol supplement (5 g pure cholesterol/kg vs. 5 g oxidized cholesterol/kg), the dietary fat (coconut oil vs. salmon oil) and the vitamin E concentration of the diet (40 vs. 240 mg α-tocopherol equivalents per kg) were varied. Oxidized cholesterol was prepared by heating cholesterol (Sigma-Aldrich, Steinheim, Germany) placed as a thin film on a glass Petri dish at 115°C for 48 hours in an electric oven. To equalize the vitamin E concentrations of the diets irrespective of the dietary fats used, the native tocopherol concentrations of the two fats were analysed. Based on the native concentrations of the fats, diets were supplemented individually with all-rac-α-tocopheryl acetate, allowing for a biopotency of 67% compared to α-tocopherol. The fatty acid composition of coconut oil (Palmin, Hamburg, Germany) and salmon oil (Caelo, Hilden, Germany) are shown in Tab. 1. The peroxide values of the salmon oil and the coconut oil were 3.9 and <0.1 mEq $O_2$ per kg fat, respectively.

**Tab. 1.** Fatty acid composition of the dietary fats[*]

| Fatty acid | Coconut oil | Salmon oil |
|---|---|---|
| | *g/100g fatty acids* | |
| Total saturated fatty acids | 91.2 | 27.1 |
| Total monounsaturated fatty acids | 6.4 | 39.5 |
| Total polyunsaturated fatty acids | 2.0 | 29.1 |
|    (n-6) polyunsaturated fatty acids | 1.9 | 3.1 |
|    (n-3) polyunsaturated fatty acids | 0.1 | 23.8 |
| Unsaturation index[‡] | 0.10 | 1.73 |

[*]Fatty acids in quantities <0.05 g/100 g were not considered.
[‡]Average number of double bonds per mol of fatty acids

The diets were prepared weekly by mixing the fat with the dry components and water. Afterwards the diets were freeze dried and stored at –20°C to prevent autoxidation of lipids, e.g. PUFAs and cholesterol. The water content after freeze drying was below 5 g per 100 g of diet. To standardize the diet intake, the diets were fed daily in restricted amounts at 0800 h. The feeding schedule was identical in both experiments. The diet intake was recorded daily and increased from 7.0 g/d to 15.0 g/g during the experiment, resulting in an average daily diet intake of 14.4 g per rat in both experiments. Water was available ad libitum from nipple drinkers. The experimental diets were fed for 35 d in both experiments.

## 2.3 Sample collection

After 35 days of feeding the experimental diets the rats were starved overnight, anesthetized with diethyl ether and killed by decapitation. Blood was collected into heparinized polyethylene tubes (Sarstedt, Nürnbrecht, Germany). The liver was excised immediately and frozen with liquid nitrogen. Plasma was separated from blood by centrifugation (1100 g, 10 min) at 4°C and stored at –80°C. Erythrocytes were washed three times with physiological saline by centrifugation and resuspension. Lipoproteins were isolated by sequential

ultracentrifugation (900,000 g, 1.5 h) at 4°C. Until analysis, plasma, LDL, erythrocyte membrane, and liver samples were stored at –80°C.

**2.4 Analyses**
The fatty acid composition of the experimental fats was determined by gas chromatography of fatty acid methyl esters [23].

Liver, plasma, LDL and erythrocyte 7β-OH-Chol (Sigma-Aldrich) and oxysterol concentration of the diets were determined using a quantitative gas chromatography-mass spectrometry (GC-MS) method with selective ion monitoring [24]. Liver and erythrocyte lipids were extracted with a mixture of hexane and isopropanol (3:2, v/v) [25]. After aliquots of the liver and erythrocyte lipid extracts, respectively, were dried under a stream of nitrogen, 2 ml of 1 M potassium hydroxide solution and 5α-cholestane (Sigma-Aldrich) as internal standard were added and the tubes flushed with nitrogen. To 0.5 ml of plasma and 0.25 ml of LDL fraction, 2 ml of 1 M potassium hydroxide solution and 5α-cholestane were directly added. After saponification overnight in the dark, double-distilled water was added and lipids were extracted with (peroxide-free) diethyl ether. The lipid extract was dried under a stream of nitrogen and then derivatized with *bis*-(trimethyl-silyl)-trifluoroacetamide (BSTFA, Sigma-Aldrich) and pyridine by heating samples at 60°C for 60 min. A Shimadzu QP-5000 GC-MS (Shimadzu, Duisburg, Germany) fitted with a DB 5 fused silica column (30 m, 0.25 mm internal diameter, 0.25 μm film thickness, J&W Scientific, Folsom, CA) was used for GC-MS analysis operating with selected ion monitoring. Helium was used as the carrier gas with a flow rate of 1 ml/min (40 cm/s). 1 μl of the samples was injected with a split ratio of 1:20. Peak identification was performed by retention time and ion fragmentation comparison with external standards. Two characteristic ions per substance were used for each oxysterol. The quantification was conducted with calibration curves calculated with the internal standard. In order to control for the formation of 7β-OH-Chol during sample treatment we ran samples with cholesterol only. The results indicated no significant formation of 7β-OH-Chol during sample treatment. We further ran samples with spiked amounts of the oxysterols determined in the diets to gain insight into the stability of those oxysterols during the whole sample work-up. We found recoveries of more than 95% for those oxysterols indicating a high stability and low degradation during sample treatment.

Total cholesterol concentrations of liver, erythrocytes, plasma, and LDL were determined using an enzymatic reagent kit obtained from Merck Eurolab (Darmstadt, Germany). For the measurement of liver and erythrocyte total cholesterol, lipids of the extract were dissolved in Triton X-100 before enzymatic measurement as described by De Hoff et al. [26].

Concentrations of individual tocopherols in plasma, liver, LDL, and dietary fats were determined using a high-performance liquid chromatography (HPLC) method [27]. After saponification with saturated sodium hydroxide solution, individual tocopherols were extracted with n-hexane and separated isocratically on a HP 1100 HPLC station (Hewlett Packard, Waldbronn, Deutschland) using a mixture of n-hexane and 1,4 dioxane (96:4, v/v) as mobile phase and a LiChrosorb Si 60 column (5 μm particle size, 250 mm length, 4 mm internal diameter, Merck Eurolab) and detected by fluorescence.

**2.5 Statistics**
Data were subjected to ANOVA using the Minitab Statistical Software (Minitab, State College, PA, USA). In Experiment 1, the factors dietary fat (coconut oil vs. salmon oil), dietary vitamin E (240 vs. 40 mg/kg diet) and the interactions of those factors were included. In Experiment 2, ANOVA was performed separately for the rats fed coconut oil and for the rats fed salmon oil. Classification factors were cholesterol supplement (pure cholesterol vs. oxidized cholesterol), dietary vitamin E (240 mg vs. 40 mg/kg diet) and the interactions

between those factors. In the case of large differences in the variances of means, data were transferred into their logarithms prior to ANOVA. For statistically significant F values, individual means of the treatment groups were compared by Fisher´s multiple range test. Means were considered significantly different for $P<0.05$.

# 3 Results
## 3.1 Experiment 1
The growth of the rats was significantly influenced by the dietary fat. Animals whose diets contained salmon oil gained significantly more body weight than the animals whose diets contained coconut oil. Average daily body weight gains were $5.6 \pm 0.4$ g and $5.9 \pm 0.3$ g (means $\pm$ SD, n=18 for each dietary fat, $P<0.05$), that is for the rats fed coconut oil and the rats fed salmon oil. The dietary vitamin E concentration had no effect on the growth of the rats.

Among the various tocopherols, only α-tocopherol was detected in considerable concentrations in all analysed samples. The concentrations of α-tocopherol in liver, plasma and LDL were significantly influenced by dietary fat and dietary vitamin E concentration (Tab. 2). Rats fed the coconut oil diets had higher concentrations of α-tocopherols in those samples than rats fed the salmon oil diets. Increasing the dietary vitamin E concentration from 40 to 240 mg α-tocopherol equivalents/kg caused a significant increase of the α-tocopherol concentration in liver, plasma and LDL. There were, however, significant interactions between the dietary fat and the dietary vitamin E concentration regarding the α-tocopherol concentration in liver and LDL. The effect of the dietary vitamin E on the α-tocopherol concentration of the liver was stronger in rats fed coconut oil than in rats fed salmon oil; the effect of the dietary vitamin E on the α-tocopherol concentrations in LDL, in the opposite, was stronger in rats fed salmon oil than in the rats fed coconut oil. The effect of the dietary vitamin E concentration on the α-tocopherol concentration in plasma was independent of the dietary fat.

**Tab 2.** Concentrations of total tocopherols, total cholesterol and 7β-hydroxycholesterol in samples of rats fed diets with coconut oil or salmon oil and vitamin E concentrations of 40 or 240 mg/kg (Experiment 1)[†]

| Oil | Coconut oil | | Salmon oil | | P values | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Vitamin E (mg/kg) | 40 | 240 | 40 | 240 | fat | vitamin E | fat x vitamin E |
| Total tocopherols | | | | | | | |
| Liver (nmol/g) | $114 \pm 22^b$ | $352 \pm 79^a$ | $54 \pm 15^c$ | $145 \pm 45^b$ | 0.001 | 0.001 | 0.001 |
| Plasma (μmol/L) | $20 \pm 2^c$ | $35 \pm 10^a$ | $15 \pm 2^d$ | $28 \pm 3^b$ | 0.01 | 0.001 | NS |
| LDL (μmol/L) | $6 \pm 1^c$ | $11 \pm 1^b$ | $8 \pm 2^c$ | $18 \pm 5^a$ | 0.001 | 0.001 | 0.05 |
| Total cholesterol | | | | | | | |
| Liver (μmol/g) | $9 \pm 1^c$ | $9 \pm 1^c$ | $16 \pm 4^a$ | $13 \pm 2^b$ | 0.001 | NS | NS |
| Plasma (mmol/L) | $1.5 \pm 0.2^c$ | $1.5 \pm 0.2^c$ | $1.7 \pm 0.2^b$ | $1.9 \pm 0.4^a$ | 0.001 | NS | NS |
| LDL (mmol/L) | $0.22 \pm 0.07^b$ | $0.21 \pm 0.08^b$ | $0.55 \pm 0.25^a$ | $0.62 \pm 0.28^a$ | 0.001 | NS | NS |
| Erythrocytes (μmol/g) | $3.5 \pm 1.0^b$ | $4.0 \pm 0.3^{ab}$ | $4.2 \pm 0.5^{ab}$ | $4.5 \pm 0.2^a$ | 0.01 | NS | NS |
| 7β-hydroxycholesterol | | | | | | | |
| Liver (nmol/g) | $1.4 \pm 0.7^b$ | $1.7 \pm 0.8^b$ | $4.4 \pm 3.0^a$ | $3.5 \pm 3.2^a$ | 0.05 | NS | NS |
| Plasma (nmol/L) | $98 \pm 22^c$ | $66 \pm 9^c$ | $232 \pm 61^a$ | $130 \pm 24^b$ | 0.001 | 0.001 | 0.01 |
| LDL (nmol/L) | $14 \pm 7^b$ | $17 \pm 6^b$ | $38 \pm 6^a$ | $33 \pm 5^a$ | 0.001 | NS | NS |
| Erythrocytes (nmol/g) | $0.15 \pm 0.02^b$ | $0.17 \pm 0.03^b$ | $0.28 \pm 0.07^a$ | $0.29 \pm 0.04^a$ | 0.001 | NS | NS |

[†]Results are means $\pm$ SD, n=9 for each treatment group. Values within one sample without a common superscript letter differ, $P<0.05$. NS - non significant.

The concentration of total cholesterol in liver, plasma, LDL and erythrocytes were significantly influenced by the dietary fat (Tab. 2). Animals whose diets contained salmon oil had significantly higher concentrations of total cholesterol in all those samples than the animals whose diets contained coconut oil. The dietary vitamin E concentration had no significant effect on the concentration of total cholesterol in the analyzed samples.

The concentrations of 7β-OH-Chol in liver, LDL and erythrocytes were significantly influenced by the dietary fat but not by the dietary vitamin E concentration. Rats whose diets contained salmon oil had significantly higher concentrations of 7β-OH-Chol in those samples than the animals whose diets contained coconut oil. The concentration of 7β-OH-Chol in plasma was not only significantly influenced by the dietary fat but also by the dietary vitamin E concentration; a significant interaction between both factors occurred there as well. Rats fed the salmon oil diets had significantly higher concentrations of 7β-OH-Chol in plasma than rats fed the coconut oil diet. Increasing the dietary vitamin E concentration from 40 to 240 mg α-tocopherol equivalents/kg significantly reduced the concentration of 7β-OH-Chol in plasma. The effect of the dietary vitamin E was, however, stronger in the rats fed the salmon oil diet than in rats fed the coconut oil diet.

## 3.2 Experiment 2

The diets supplemented with 5 g oxidized cholesterol/kg contained the following oxysterols (mg/kg): 7β-OH-Chol (2.7), α-epoxycholesterol (2.6), β-epoxycholesterol (6.9), cholestanetriol (0.1), 25-hydroxycholesterol (9.4), 7-ketocholesterol (1.7). The concentration of cholesterol was 4.63 g/kg diet in the diet supplemented with oxidized cholesterol. Other unidentified oxysterols have not been quantified. In the diets containing pure cholesterol the concentrations of all oxysterols were below the limit of detection of 0.025 mg/kg.

The average daily body weight gains of the rats in Experiment 2 were similar to those in Experiment 1 with 5.5 ± 0.3 g for the rats fed coconut oil and 6.0 ± 0.3 g for the rats fed salmon oil (means ± SD, n=36 for each dietary fat). The type of cholesterol and the dietary vitamin E concentration did not influence body weight gains of the rats fed both types of fat.

As in Experiment 1, among the various tocopherol isomers, α-tocopherol was the only one existing in considerable concentrations in all analysed samples. In the rats fed both types of fat, increasing the dietary vitamin E concentration from 40 to 240 mg α-tocopherol equivalents/kg led to a significant increase of the concentration of α-tocopherol in liver, plasma and LDL (Tab. 3). In the rats fed the salmon oil diets, the concentration of α-tocopherol in plasma and LDL was also influenced by the type of dietary cholesterol. Rats fed the diets containing oxidized cholesterol had lower concentrations of α-tocopherol in plasma and LDL than rats fed the diets containing pure cholesterol; the concentration of α-tocopherol in the liver was independent of the type of dietary cholesterol. In the rats fed the coconut oil diets, the type of cholesterol did not influence the concentration of α-tocopherol in any of the tissues investigated.

**Tab 3.** Concentrations of total tocopherols in samples of rats fed diets with pure cholesterol or oxidized cholesterol at two different dietary fats (coconout oil vs. salmon oil) and two different vitamin E concentrations (40 vs.240 mg/kg) (Experiment 2)[†]

| Cholesterol | Cholesterol | | Oxidized cholesterol | | P values | | |
| Vitamin E (mg/kg) | 40 | 240 | 40 | 240 | cholesterol | vitamin E | cholesterol x vitamin E |
|---|---|---|---|---|---|---|---|
| | | | Coconut oil | | | | |
| Liver (nmol/g) | $125 \pm 40^b$ | $545 \pm 92^a$ | $163 \pm 37^b$ | $534 \pm 159^a$ | NS | 0.001 | NS |
| Plasma (μmol/L) | $29 \pm 7^b$ | $50 \pm 7^a$ | $27 \pm 2^b$ | $47 \pm 9^a$ | NS | 0.001 | NS |
| LDL (μmol/L) | $11 \pm 4^b$ | $16 \pm 4^a$ | $9 \pm 3^b$ | $15 \pm 5^a$ | NS | 0.001 | NS |
| | | | Salmon oil | | | | |
| Liver (nmol/g) | $96 \pm 21^c$ | $434 \pm 83^a$ | $83 \pm 16^c$ | $360 \pm 96^b$ | NS | 0.001 | NS |
| Plasma (μmol/L) | $11 \pm 2^c$ | $24 \pm 4^a$ | $9 \pm 2^c$ | $19 \pm 5^b$ | 0.01 | 0.001 | NS |
| LDL (μmol/L) | $7 \pm 1^b$ | $15 \pm 3^a$ | $5 \pm 1^b$ | $12 \pm 4^a$ | 0.05 | 0.001 | NS |

[†]Results are means ± SD, n=9 for each treatment. Values within a row without a common superscript letter differ, *P*<0.05. NS - non significant.

In the rats fed the coconut oil diets, the concentration of total cholesterol in all the analysed tissues was not influenced by the type of dietary cholesterol and the dietary vitamin E concentration (Tab. 4). In the rats fed the salmon oil diets, dietary oxidized cholesterol significantly reduced the concentrations of total cholesterol in plasma and erythrocytes as compared with pure cholesterol; the cholesterol concentrations in liver and LDL were not influenced by the type of dietary cholesterol. In the rats fed the salmon oil diets, the dietary vitamin E concentration also had no effect on the cholesterol concentration in any of the analysed samples.

**Tab 4.** Concentrations of total cholesterol in samples of rats fed diets with pure cholesterol or oxidized cholesterol at two different dietary fats (coconout oil vs. salmon oil) and two different vitamin E concentrations (40 vs.240 mg/kg) (Experiment 2)[†]

| Cholesterol | Cholesterol | | Oxidized cholesterol | | P values | | |
| Vitamin E (mg/kg) | 40 | 240 | 40 | 240 | cholesterol | vitamin E | cholesterol x vitamin E |
|---|---|---|---|---|---|---|---|
| | | | Coconut oil | | | | |
| Liver (μmol/g) | $103 \pm 29$ | $101 \pm 21$ | $111 \pm 25$ | $104 \pm 23$ | NS | NS | NS |
| Plasma (mmol/L) | $4.8 \pm 0.5$ | $4.7 \pm 0.7$ | $4.5 \pm 0.6$ | $4.4 \pm 0.9$ | NS | NS | NS |
| LDL (mmol/L) | $0.67 \pm 0.27$ | $0.65 \pm 0.31$ | $0.85 \pm 0.42$ | $0.77 \pm 0.34$ | NS | NS | NS |
| Erythrocytes (μmol/g) | $4.0 \pm 0.4$ | $3.8 \pm 0.8$ | $4.3 \pm 0.6$ | $3.6 \pm 0.6$ | NS | NS | NS |
| | | | Salmon oil | | | | |
| Liver (μmol/g) | $125 \pm 22$ | $135 \pm 32$ | $133 \pm 25$ | $142 \pm 24$ | NS | NS | NS |
| Plasma (mmol/L) | $1.9 \pm 0.3^{ab}$ | $2.3 \pm 0.4^a$ | $1.7 \pm 0.2^b$ | $1.9 \pm 0.5^b$ | 0.01 | NS | NS |
| LDL (mmol/L) | $0.43 \pm 0.10$ | $0.47 \pm 0.19$ | $0.39 \pm 0.121$ | $0.33 \pm 0.13$ | NS | NS | NS |
| Erythrocytes (μmol/g) | $4.4 \pm 0.4^{ab}$ | $4.5 \pm 0.5^a$ | $3.9 \pm 0.6^b$ | $3.7 \pm 0.9^b$ | 0.01 | NS | NS |

[†]Results are means ± SD, n=9 for each treatment. Values within a row without a common superscript letter differ, *P*<0.05. NS - non significant.

In the rats fed both types of fats, the concentrations of 7β-OH-Chol in liver, LDL and erythrocytes were, as in Experiment 1, independent of the dietary vitamin E concentration (Tab. 5). In contrast, the concentration of 7β-OH-Chol in plasma was significantly reduced by increasing the dietary vitamin E concentration from 40 to 240 mg α-tocopherol equivalents/kg in the rats fed both types of fat. In the rats fed the coconut oil diets, oxidized cholesterol caused a significant increase of the concentration of 7β-OH-Chol in all the tissues analysed as compared with pure cholesterol. In the rats fed the salmon oil diets, dietary oxidized

cholesterol increased the concentrations of 7β-OH-Chol in liver and plasma; the concentrations of 7β-OH-Chol in LDL and erythrocytes were not influenced by the type of dietary cholesterol.

**Tab 5.** Concentrations of 7β-hydroxycholesterol in samples of rats fed diets with pure cholesterol or oxidized cholesterol at two different dietary fats (coconout oil vs. salmon oil) and two different vitamin E concentrations (40 vs.240 mg/kg) (Experiment 2)[†]

| Cholesterol | Cholesterol | | Oxidized cholesterol | | P values | | |
|---|---|---|---|---|---|---|---|
| Vitamin E (mg/kg) | 40 | 240 | 40 | 240 | cholesterol | vitamin E | cholesterol x vitamin E |
| Coconut oil | | | | | | | |
| Liver (nmol/g) | $7 \pm 2^b$ | $9 \pm 3^b$ | $14 \pm 2^a$ | $14 \pm 5^a$ | 0.001 | NS | NS |
| Plasma (nmol/L) | $374 \pm 122^c$ | $206 \pm 92^d$ | $770 \pm 90^a$ | $500 \pm 110^b$ | 0.001 | 0.001 | NS |
| LDL (nmol/L) | $63 \pm 17^b$ | $60 \pm 11^b$ | $94 \pm 10^a$ | $96 \pm 32^a$ | 0.05 | NS | NS |
| Erythrocytes (nmol/g) | $0.24 \pm 0.07^{ab}$ | $0.20 \pm 0.10^b$ | $0.31 \pm 0.07^a$ | $0.33 \pm 0.12^a$ | 0.01 | NS | NS |
| Salmon oil | | | | | | | |
| Liver (nmol/g) | $26 \pm 4^b$ | $21 \pm 3^b$ | $40 \pm 13^a$ | $39 \pm 13^a$ | 0.001 | NS | NS |
| Plasma (nmol/L) | $558 \pm 136^b$ | $326 \pm 78^c$ | $707 \pm 156^a$ | $497 \pm 176^b$ | 0.01 | 0.001 | NS |
| LDL (nmol/L) | $99 \pm 22^{ab}$ | $74 \pm 6^b$ | $120 \pm 26^a$ | $94 \pm 15^{ab}$ | NS | NS | NS |
| Erythrocytes (nmol/g) | $0.32 \pm 0.06$ | $0.28 \pm 0.10$ | $0.33 \pm 0.20$ | $0.34 \pm 0.10$ | NS | NS | NS |

[†]Results are means ± SD, n=9 for each treatment. Values within a row without a common superscript letter differ, $P<0.05$. NS - non significant.

## 4. Discussion

The primary aim of this study was to investigate the effects of dietary PUFA, oxidized cholesterol and vitamin E on the concentration of 7β-OH-Chol in rat tissues. Implementation of a restrictive feeding regime enabled us to standardize the feed intake, thus eliminating secondary effects which might result from differences in the feed intake. In both experiments, the animals whose diet contained salmon oil unexpectedly gained more weight than the animals whose diet contained coconut oil. This effect might be due to the low levels of linoleic and α-linolenic acid in coconut oil. There were, however, no signs of a deficiency of essential fatty acids, as evidenced by the low concentration of 20:3 (n-9) in the erythrocyte membrane. We do not consider that the lower weight gains of the rats whose diet contained coconut oil jeopardize the results of the study as a whole.

One main finding of this study was that feeding a diet containing fish oil leads to higher concentrations of 7β-OH-Chol in liver, plasma, LDL, and erythrocytes as compared with feeding a diet containing coconut oil. Increased concentrations of 7β-OH-Chol in liver, plasma and LDL of rats fed fish oil compared with rats fed coconut oil, observed in Experiment 1, might be partially due to the fact that fish oil feeding increased the concentrations of total cholesterol in those samples. The higher hepatic cholesterol concentration in rats fed salmon oil compared with rats fed coconut oil might be due to an increased activity of acyl CoA:cholesterol acyl transferase caused by fish oil [28, 29]. The main reason for increased concentrations of 7β-OH-Chol may be that feeding fish oil causes an incorporation of highly unsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid in tissues and lipoproteins, which are highly susceptible to lipid peroxidation [16, 17]. We assume that in rats fed salmon oil the oxidation of cholesterol in liver, lipoproteins and erythrocyte membranes was promoted by an increased rate of oxidation of PUFA. In a recent study with rats, we also found higher concentrations of 7β-OH-Chol and other oxysterols in the liver of rats fed salmon oil than in rats fed lard [30].

The vitamin E requirement of man and animals depends mainly on the intake of dietary PUFA. Assuming a specific vitamin E requirement for unsaturated fatty acids as

suggested by Muggli [31], we estimated the vitamin E requirement of the rats on the salmon oil diets at 51 mg all-*rac*-α-tocopheryl acetate/kg diet, and that of the rats on the coconut oil diets at 3 mg all-*rac*-α-tocopheryl acetate/kg diet. As expected, increasing the dietary vitamin E concentration from 40 to 240 mg per kg markedly increased the concentration of α-tocopherol in the tissues investigated. Lower tocopherol concentrations, after feeding the salmon oil diets as compared with feeding coconut oil, are due to enhanced use and depletion of vitamin E by highly unsaturated PUFA [16, 17]. The finding that feeding oxidized cholesterol reduces the concentrations of α-tocopherol in plasma and LDL in rats fed salmon oil as compared with feeding pure cholesterol could be the result of oxidative stress due to oxidized cholesterol. There is some indication from *in vitro* studies that oxidized cholesterol is a source of oxidative stress [32, 33]. Increased concentrations of α-tocopherol in plasma and LDL of rats fed the coconut oil diets supplemented with pure or oxidized cholesterol in Experiment 2 relative to rats fed the coconut oil diets without cholesterol in Experiment 1 may be due to their higher lipid concentrations in plasma and LDL. A strong relationship exists between the concentration of tocopherols and the concentration of total lipids in plasma [34]. The finding that an increase of the dietary vitamin E concentration from 40 to 240 mg/kg failed to reduce the concentrations of 7β-OH-Chol in liver, LDL and erythrocytes suggests that a dietary vitamin E concentration of 40 mg/kg provided maximum protection of cholesterol in these samples against oxidation even in the case of fish oil as dietary fat. The observation that increased dietary vitamin E reduced the concentrations of 7β-OH-Chol in plasma but not in the liver suggests that vitamin E is able to prevent cholesterol in lipoproteins against oxidative modification during their circulation in the blood. However, because the concentration of 7β-OH-Chol in LDL was not reduced by increased dietary vitamin E concentrations, vitamin E may have protected cholesterol only in very low density lipoproteins or high density lipoproteins. The role of vitamin E in the protection of cholesterol against oxidation in lipoproteins requires further investigation.

Comparing the concentrations of 7β-OH-Chol in the liver as found in Experiment 1 with those found in Experiment 2 shows that feeding a diet enriched with cholesterol largely enhances the formation of 7β-OH-Chol in the liver. This is probably due to cholesterol accumulation in the liver by cholesterol feeding. Other studies also demonstrated that there is a relationship between the concentration of cholesterol and the concentrations of 7β-OH-Chol in the liver [35, 36]. Oxidative modification of cholesterol is favoured by free radicals released from the autoxidation of PUFA as shown in *in vitro* studies with phospholipid membrane bilayers [14, 15]. This might explain why simultaneous feeding of salmon oil strongly enhanced the formation of 7β-OH-Chol in the liver of rats fed cholesterol.

In Experiment 2, diets were supplemented with an oxidized cholesterol preparation. To provide a rough estimate of the composition and the degree of oxidation of the oxidized cholesterol preparation used in this study we measured six of the dominating oxysterols knowing to be formed under the applied oxidizing conditions and cholesterol. Cholesterol contributed to about 93% in the oxidized cholesterol preparation. This indicates that we, in contrast to other investigators using oxidized cholesterol preparations which were almost completely oxidized, used a moderately oxidized cholesterol preparation [35, 36]. This is by far more relevant with respect to human diets concerning the estimation that about 1% of the cholesterol consumed in a mixed Western diet is oxidized [37]. The study showed that the concentration of 7β-OH-Chol in the liver was significantly increased by feeding oxidized cholesterol as compared with feeding pure cholesterol. This demonstrates that dietary oxysterols like 7β-OH-Chol are incorporated into the liver. Other studies also reported increased concentrations of hepatic oxysterols like 7β-OH-Chol after feeding diets supplemented with oxidized cholesterol [21, 35, 36]. It was remarkable that the concentration of 7β-OH-Chol in erythrocyte membranes, in opposite to those in liver and plasma, was not significantly increased by feeding diets with oxidized cholesterol. This suggests that less

oxidative modified cholesterol was transferred from lipoproteins to erythrocytes during the renewal of the erythrocyte membrane. Relatively low concentrations of 7β-OH-Chol in erythrocytes, when expressed per mmol of cholesterol, generally suggest that erythrocytes in comparison to liver or plasma are well protected against oxidative modification of cholesterol located within the membrane.

It has been shown that hepatic oxysterols are incorporated into lipoproteins [38, 39]. We, therefore, assume that most of 7β-OH-Chol present in plasma and lipoproteins derives mainly from the liver. Hence, it was not surprising that the concentrations of 7β-OH-Chol in plasma and LDL, when related to the concentration of total cholesterol, within the treatment groups reflected those of the liver. The contribution of dietary oxysterols to the development of atherosclerosis is still discussed controversially [21, 40]. The present study, however, suggests that dietary regimes rich in oxysterols could be critical because they increase the concentration of 7β-OH-Chol in LDL.

From view of pathophysiology, the 7β-OH-Chol concentrations in plasma and LDL might be relevant. It has been shown that 7β-OH-Chol in plasma and lipoproteins has cytotoxic effects on cells of the arterial wall [3]. Plasma concentrations of 7β-OH-Chol have been used as a possible marker of oxidative stress [4, 5]. However, it is not clear whether raised 7β-OH-Chol will increase the risk of atherosclerosis. Although the results found in rats cannot be directly transferred to humans due to a different lipoprotein profile and lipoprotein metabolism (smaller LDL fraction and lacking of cholesterol ester transfer protein in rats), the study suggests that consumption of fish oil, with respect to the formation of 7β-OH-Chol, must be considered critically. Highly unsaturated fatty acids enhance the formation of 7β-OH-Chol in the liver which is secreted into the blood by lipoproteins. Diet regimes with high concentrations of 7β-OH-Chol must be considered cautiously regarding the potential incorporation of dietary 7β-OH-Chol into plasma lipoproteins. Supplementation of vitamin E above the animals´ requirement reduced the concentrations of 7β-OH-Chol only in plasma but not in liver, LDL and erythrocytes and thus did not provide an efficient protection against enhanced formation of 7β-OH-Chol by dietary fish oil, cholesterol and oxidized cholesterol.

# References

[1] *S. M. Colles, J. M. Maxson, S. G. Carlson, S.G., G. M. Chisolm:* Oxidized LDL-induced injury and apoptosis in atherosclerosis. Potential roles for oxysterols. Trends Cardiovasc. Med. **11** (2001) 131-138.

[2] *K. L. H. Carpenter, S. E. Taylor, J. A. Ballantine, B. Fussell, B. Halliwell, M. J. Mitchinson:* Lipids and oxidized lipids in human atheroma and normal aorta. Biochim. Biophys. Acta **1167** (1993) 121-130.

[3] *G. Lizard, S. Monier, C. Cordolet, L. Gesquiére, V. Deckert, S. Gueldry, L. Lagrost, P. Gambert:* Characterization and comparison of the mode of cell death, apotosis versus necrosis, induced by 7β-hydroxycholesterol and 7-ketocholesterol in the cells of the vascular wall. Arterioscler. Thromb. Vasc. Biol. **19** (1999) 1190-1200.

[4] *J. T. Salonen, K. Nyyssönen, R. Salonen, E. Porkkala-Sarataho, T.-P. Tuomainen, U. Diczfalusy, I. Björkhem:* Lipoprotein oxidation and progression of carotid atherosclerosis. Circulation **95** (1997) 840-845.

[5] *B. Zieden, A. Kaminskas, M. Kristenson, Z. Kucinskiene, B. Vessby, A. G. Olsson, U. Diczfalusy:* Increased plasma 7β-hydroxycholesterol concentrations in a population with a high risk for cardiovascular disease. Atheroscler. Thromb. Vasc. Biol. **19** (1999) 967-971.

[6] *H. N. Hodis, A. Chauhan, S. Hashimoto, D. W. Crawford, A. Sevanian:* Probucol reduces plasma and aortic wall oxysterol levels in cholesterol fed rabbits independently of its plasma cholesterol lowering effect. Atherosclerosis **96** (1992) 125-134.

[7] *I. Björkhem:* Do oxysterols control cholesterol homeostasis? J. Clin. Invest. **110** (2002) 725-730.

[8] *H. A. Emanuel, C. A. Hassel, P. B. Addis, S. D. Bergmann, J. H. Zavoral:* Plasma cholesterol oxidation products (Oxysterols) in human subjects fed a meal rich in oxysterols. J. Food Sci. **56** (1991) 843-847.

[9] *J. Linseisen, G. Wolfram:* Absorption of cholesterol oxidation products from ordinary foodstuffs in humans. Ann. Nutr. Metab. **42** (1998) 221-230.

[10] *K. Ariyoshi, J. Adachi, M. Asano, Y. Ueno, R. Rajendram, V. R. Preedy:* Effect of chronic ethanol feeding on oxysterols in rat liver. Free Radic. Res. **36** (2002) 661-666.

[11] *J. Adachi, R. Kudo, Y. Ueno, R. Hunter, R. Rajendram, E. Want, V. R. Preedy:* Heart 7-hydroperoxycholesterol and oxysterols are elevated in chronically ethanol-fed rats. J. Nutr. **131** (2001) 2916-2920.

[12] *M. M. Mahfouz, F. A. Kummerow:* Cholesterol-rich diets have different effects on lipid peroxidation, cholesterol oxides, and antioxidant enzymes in rats and rabbits. J. Nutr. Biochem. **11** (2000) 293-302.

[13] *H. N. Hodis, D. W. Crawford, A. Sevanian:* Cholesterol feeding increases plasma and aortic tissue cholesterol oxide levels in parallel: further evidence for the role of cholesterol oxidation in atherosclerosis. Atherosclerosis **89** (1991) 117-126.

[14] *A. Sevanian, L. L. McLeod:* Cholesterol autoxidation in phospholipid membrane bilayers. Lipids **22** (1987) 627-636.

[15] *J. Terao, K. Sugino, S. Matsushita:* $Fe^{2+}$ and ascorbic acid induced oxidation of cholesterol in phosphatidylcholine liposomes and its inhibition by alpha-tocopherol. J. Nutr. Sci. Vitaminol. **31** (1985) 499-508.

[16] *S.-H. Cho, Y.-S. Choi:* Lipid peroxidation and antioxidant status is affected by different vitamin E levels when feeding fish oil. Lipids **29** (1994) 47-52.

[17] *M.-L. Hu, E. N. Frankel, B. E. Leibovitz, A. L. Tappel:* Effect of dietary lipids and vitamin E on in vitro lipid peroxidation in rat liver and kidney homogenates. J. Nutr. **119** (1989) 1574-1582.

[18] *M. M. Mahfouz, H. Kawano, F. A. Kummerow:* Effect of cholesterol-rich diets with and without added vitamins E and C on the severity of atherosclerosis in rabbits. Am. J. Clin. Nutr. **66** (1997) 1240-1249.

[19] *M. J. T. M. Mol, Y. B. de Rijke, P. N. M. Demaker, A. F. H. Stalenhoef:* Plasma levels and cholesterol oxidation products and cytokines in diabetic mellitus and cigarette smoking: effects of vitamin E treatment. Atherosclerosis **129** (1997) 169-176.

[20] *D. F. Vine, K. D. Croft, L. J. Beilin, J. C. L. Mamo:* Absorption of dietary cholesterol oxidation products and incorporation into rat lymph chylomikrons. Lipids **32** (1997) 887-893.

[21] *I. Staprans, X.-M. Pan, J. H. Rapp, K. R. Feingold:* Oxidized cholesterol in the diet accelerates the development of aortic atherosclerosis in cholesterol-fed rabbits. Arterioscler. Thromb. Vasc. Biol. **18** (1998) 977-983.

[22] *P. G. Reeves, F. H. Nielsen, G. C. Fahey:* AIN-93 Purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc Writing Committee on the Reformulation of the AIN-76A Rodent Diet. J. Nutr. **123** (1993) 1939-1951.

[23] *S. Daenicke, H. Jeroch, O. Simon, M. R. Bedford:* Interactions between dietary fat type and exogenous enzyme supplementation of broiler diets based on maize, wheat, triticale or barley. J. Anim. Feed Sci. **8** (1999) 467-483.

[24] *T. A. Mori, K. D. Croft, I. B. Puddey, L. J. Beilin:* Analysis of native and oxidized low-density lipoprotein oxysterols using gas chromatography-mass spectrometry with selective ion monitoring. Redox Report **2** (1996) 25-34.

[25] *A. Hara, N. S. Radin:* Lipid extraction of tissues with a low toxicity solvent. Anal. Biochem. **90** (1978) 420-426.

[26] *J. L. De Hoff, J. H. Davidson, V. Kritchevsky:* An enzymatic assay for determining free and total cholesterol in tissues. Clin. Chem. **24** (1978) 433-435.

[27] *M. K. Balz, E. Schulte, H. P. Their:* Simultaneous determination of tocopherol acetate, tocopherols and tocotrienols by HPLC with fluorescence detection in foods. Fat Sci. Technol. **95** (1993) 215-220.

[28] *M. Chautan, F. Chanussot, H. Portugal, A. M. Pauli, H. Lafont:* Effects of salmon oil and corn oil on plasma lipid level and hepato-biliary cholesterol metabolism in rats. Biochim. Biophys. Acta. **1046** (1990) 40-45.

[29] *F. J. Field, E. J. Albright, S. N. Mathur:* Effect of dietary n-3 fatty acids on HMG-CoA reductase and ACAT activities in liver and intestine of the rabbit. J. Lipid Res. **28** (1987) 50-58.

[30] *C. Brandsch, R. Ringseis, K. Eder:* High dietary iron concentrations enhance the formation of cholesterol oxidation products in the liver of adult rats fed salmon oil with minimal effects on the antioxidant status. J. Nutr. **132** (2002) 2263-2269.

[31] *R. Muggli:* Polyunsaturated fatty acids and the requirement of vitamin E. Fat Sci. Technol. **96** (1994) 17-19.

[32] *G. Bansal, U. Singh, M. P. Bansal:* Effect of 7β-hydroxycholesterol on cellular redox status and heat shock protein 70 expression in macrophages. Cell. Physiol. Biochem. **11** (2001) 241-246.

[33] *H. Cantwell, R. Devery:* The response of the antioxidant defense system in rat hepatocytes challenged with oxysterols is modified by Covi-ox. Cell Biol. Toxicol. **14** (1998) 401-409.

[34] *K. Eder, M. Kirchgessner:* Effect of dietary fat on susceptibility of low-density lipoproteins to lipid peroxidation in pigs. J. Anim. Physiol. Anim. Nutr. **77** (1997) 66-76.

[35] *K. Osada, T. Kodama, S. Noda, K. Yamada, M. Sugano:* Oxidized cholesterol modulates age-related change in lipid metabolism in rats. Lipids **30** (1995) 405-413.

[36] *K. Osada, T. Kodama, L. Cui, Y. Ito, M. Sugano:* Effects of dietary oxidized cholesterol on lipid metabolism in differently aged rats. Biosci. Biotech. Biochem. **58** (1994) 1062-1069.

[37]  *P. van de Bovenkamp, T. G. Kosmeijer-Schuil, M. B. Katan:* Quantification of oxysterols in dutch foods: egg products and mixed diets. Lipids **23** (1988) 1079-1085.

[38]  *D. F. Vine, J. C. L. Mamo, L. J. Beilin, T. A. Mori, K. D. Croft:* Dietary oxysterols are incorporated in plasma triglyceride-rich lipoproteins, increase their susceptibility to oxidation and increase aortic cholesterol concentration of rabbits. J. Lipid Res. **39** (1998) 1995-2004.

[39]  *S.-K. Peng, G. A. Phillips, G.-Z. Xia, R. J. Morin:* Transport of cholesterol autoxidation products in rabbit lipoproteins. Atherosclerosis **64** (1987) 1-6.

[40]  *M. A. Lyons, A. J. Brown:* 7-Ketocholesterol delivered to mice in chylomicron remnant-like particles is rapidly metabolised, excreted and does not accumulate in aorta. Biochim. Biophys. Acta **1530** (2001) 209-218.

### 3.3 Effekt von Fischöl und variierender Vitamin E-Versorgung auf die 7β-Hydroxycholesterol-Konzentrationen in der Ratte

**ABSTRACT** This study was conducted to determine the interaction between the type of dietary fat (coconut oil vs. salmon oil) and the vitamin E concentration of the diet (10 vs. 20 vs. 40 vs. 240 mg α-tocopherol equivalents (α-toc)/kg) in relation to the concentration of 7β-hydroxycholesterol (7β-OH) in liver, plasma, LDL and erythrocytes of rats. In the rats whose diet contained salmon oil, the concentration of 7β-OH was dependent on the dietary vitamin E concentration. Rats whose diet contained 10 mg α-toc/kg had significantly higher concentrations of 7β-OH in all samples studied than those whose diet contained 20, 40 or 240 mg α-toc/kg. Increasing the dietary vitamin E concentration from 40 to 240 mg α-toc/kg did not reduce the concentration of 7β-OH in any samples. In the rats whose diet contained coconut oil, the concentration of 7β-OH was independent of the dietary vitamin E concentration in all samples. The study shows that insufficient vitamin E in the diet increases the formation of 7β-OH in rats fed salmon oil, whereas a dietary vitamin E supply in excess of the requirement does not lower 7β-OH concentrations compared with an adequate vitamin E supply.

KEY WORDS: ● 7β-hydroxycholesterol ● vitamin E ● oxysterols ● fish oil ● rats

[2]Abbreviations used: α-toc, α-tocopherol equivalents; 7β-OH, 7β-hydroxycholesterol; GC-MS, gas chromatography-mass spectrometry; PUFA, polyunsaturated fatty acids.
[3]Diet composition: Experimental diets contained (g/kg): casein, 200; sucrose, 200; fat, 100; cellulose, 40; corn starch, 398; mineral mixture, 40 (consisting of calcium carbonate, 7.56; dicalcium phosphate, 8.67; potassium chloride, 6.87; sodium bicarbonate, 3.77; magnesium oxide, 1.01; ferrous sulfate hydrate, 0.116; zinc oxide, 0.038; manganese oxide, 0.016; copper sulfate pentahydrate, 0.024; calcium iodate, 0.0032; sodium selenite pentahydrate, 0.0033); vitamin mixture 20 (consisting of all-trans-retinol, 1.34 mg; cholecalciferol, 25 μg; menadion sodium bisulfite, 7.5 mg; thiamine hydrochloride, 5 mg; riboflavine, 6 mg; pyridoxine hydrochloride, 6 mg; biotin, 0.2 mg; calcium pantothenate, 15 mg; nicotinic acid, 30 mg; vitamin B12, 0.025 mg; folic acid, 2 mg; choline chloride, 1,000 mg); DL-methionine, 2.

Oxysterols, which are either ingested with the diet or formed endogenously in mammals, are of great pathophysiologic importance (1) and are involved in the pathogenesis of arteriosclerosis (2). The endogenous formation of oxysterols from cholesterol is promoted especially by peroxidation of polyunsaturated fatty acids (PUFA)[2] (3, 4). The susceptibility of membranes and lipoproteins to oxidation is determined primarily by the fatty acid composition of lipids and the concentrations of antioxidants, in particular tocopherols. Incorporation of highly unsaturated fatty acids from fish oil into lipids increases the susceptibility of tissues to oxidation, whereas an increased concentration of tocopherols lowers it (5-7). There is some evidence that the endogenous formation of oxysterols is influenced by the type of dietary fat and the ingestion of antioxidants (8-12). We are not aware of any studies investigating the interactions between the type of dietary fat and the level of the vitamin E supply in relation to the concentrations of oxysterols. The objective of our study was to determine the effect of the dietary vitamin E supply on the formation of oxysterols in tissues of rats fed fish oil or coconut oil. We considered a wide spectrum of vitamin E supply, from inadequate to excessive. Fish oil was chosen because it is often used

in the prevention of arteriosclerosis due to its many favourable properties; coconut oil was used as a control fat with a very low PUFA content. From among the oxysterols formed in the body, we considered 7β-hydroxycholesterol (7β-OH) as a marker for the formation of oxysterols. The concentration of 7β-OH in tissues reflects the endogenous, nonenzymatic formation of oxysterols fairly accurately (13, 14). We determined the concentrations of 7β-OH in plasma and LDL because this is where oxysterols are of particular pathophysiologic relevance. To gain further insight into the formation of oxysterols, we also measured the concentrations of 7β-OH in liver and erythrocytes.

## MATERIALS AND METHODS

*Animals.* Male Sprague-Dawley rats (n = 72) with an initial body weight of 63 ± 5 g (mean ± SD) were obtained from Charles River (Sulzfeld, Germany). They were assigned to eight groups of nine rats each and housed in Macrolon cages in groups of 3 rats/cage in a room maintained at a temperature of 23°C and 50-60% relative humidity with lighting from 0600 to 1800 h. All experimental procedures described followed guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt.

*Diets and feeding.* We used purified diets that were formulated according to the recommendations of the AIN (15) for rat diets.[3] The dietary fat, 100 g/kg coconut oil (Palmin, Hamburg, Germany) or 100 g/kg salmon oil (Caelo, Hilden, Germany) and the vitamin E concentration of the diet (10 vs. 20 vs. 40 vs. 240 mg α-tocopherol equivalents (α-toc)/kg) were varied according to a bifactorial design. The major fatty acids in coconut oil were (g/100 g total fatty acids): caprylic acid (8:0), 8.1; capric acid (10:0), 6.3; lauric acid (12:0), 45.6, myristic acid (14:0), 17.0; palmitic acid (16:0), 9.7; stearic acid (18:0), 4.5; oleic acid (18:1), 6.3; linoleic acid (18:2), 1.9. The major fatty acids in salmon oil were (g/100 g total fatty acids): myristic acid, 6.1; palmitic acid, 14.5; palmitoleic acid (16:1), 8.2; stearic acid, 3.0; oleic acid, 12.9; linoleic acid, 2.2; α-linolenic acid (18:3(n-3)), 0.8; eicosanoic acid (20:1), 4.3; eicosapentaenoic acid (20:5(n-3)), 12.7; docosanoic acid (22:1), 4.5; docosapentaenoic acid (22:5(n-3)), 3.1; docosahexaenoic acid (22:6(n-3)), 10.0.

Vitamin E (as all-*rac*-α-tocopheryl acetate, Merck Eurolab, Darmstadt, Germany) was added to the diets at the expense of dietary fibre. To equalize the vitamin E concentrations of the diets, the native tocopherol levels of the two fats were analysed. On the basis of the native concentrations of the fats, diets were supplemented individually with all-*rac*-α-tocopheryl acetate, allowing for a biopotency of 67% compared with α-tocopherol. The diets were prepared weekly by solubilizing the all-*rac*-α-tocopheryl acetate in the fat and mixing it with the dry components and water. The diets were freeze-dried and stored at –20°C to prevent autoxidation of lipids, e.g., PUFA. The peroxide values of the dietary fats extracted from the diets with a mixture of hexane and isopropanol (3:2, according to (16)), were determined at various times during storage of the diets. They were independent of the dietary vitamin E concentration and did not significantly increase during storage. Average peroxide values of the four vitamin E concentrations, measured according to official methods (17), were 0.8 mEq $O_2$/kg fat in the coconut oil diets and 8.6 mEq $O_2$/kg fat in the salmon oil diets. The water content after freeze drying was <5 g/100 g diet. The experimental diets were fed for 42 days. Rats consumed the experimental diets and water, from nipple drinkers, ad libitum.

*Sample collection.* After completion of the feeding period, the rats were starved overnight, anesthetized with diethyl ether and killed by decapitation. Blood was collected into heparinized polyethylene tubes (Sarstedt, Nürnbrecht, Germany). The liver was excised immediately and frozen with liquid nitrogen. Plasma was separated from blood by centrifugation (1100 X g, 10 min) at 4°C and stored at –80°C. Erythrocytes were washed three times with physiologic sodium chloride solution (9 g/L). Plasma lipoproteins were separated by step-wise ultracentrifugation (Mikro-Ultracentrifuge, Sorvall Products, Bad Homburg, Germany) at 900,000 X g at 4°C for 1.5 h. In the first step, the plasma density was

adjusted to 1006 g/L by adding 0.3 mL of a solution containg 0.195 mol/L sodium chloride and 2.44 mol/L sodium bromide to 0.6 mL of plasma. After centrifugation, the upper portion of 0.3 mL, which contained the VLDL fraction, was removed by suction and discarded. Then the density was adjusted to 1063 g/L by adding 0.3 mL of a solution containing 0.195 mol/L sodium chloride and 2.44 mol/L sodium bromide. After centrifugation, the upper portion of 0.3 mL, which contained the LDL fraction, was removed by suction and used for analysis of tocopherols and 7β-OH. Concentrations of tocopherols and 7β-OH in LDL were expressed per L of plasma. Plasma, LDL, erythrocytes and liver were stored at −80°C until analysis.

*Analytical methods.* The fatty acid composition of the experimental fats was determined by gas chromatography of fatty acid methyl esters (18, 19). Liver, plasma, LDL and erythrocyte membrane 7β-OH (Sigma-Aldrich, Steinheim, Germany) was determined using a quantitative gas chromatography-mass spectrometry (GC-MS) method with selective ion monitoring (20). Liver and erythrocyte lipids were extracted with a mixture of hexane and isopropanol (3:2, v/v) (16). After aliquots of the liver and erythrocyte lipid extracts were dried under a stream of nitrogen, 1 mol/L potassium hydroxide solution and 5α-cholestane (Sigma-Aldrich, Steinheim, Germany) as internal standard were added, respectively, and the tubes flushed with nitrogen. To 0.5 mL of plasma or 0.25 mL of LDL fraction, 1 mol/L potassium hydroxide solution and 5α-cholestane were added directly. After saponification overnight in the dark, double-distilled water was added and lipids were extracted with (peroxide-free) diethyl ether. The lipid extract was dried under a stream of nitrogen and then derivatized with *bis*-(trimethyl-silyl)-trifluoroacetamide (Sigma-Aldrich) and pyridine by heating samples at 60°C for 60 min. A Shimadzu QP-5000 GC-MS (Shimadzu, Duisburg, Germany) fitted with a DB 5 fused silica column (30 m, 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific, Folsom, CA) was used for GC-MS analysis operating with selected ion monitoring. Helium was used as the carrier gas with a flow rate of 1 mL/min (40 cm/s). The sample (1 μL) was injected with a split ratio of 1:20. Peak identification was performed by retention time and ion fragmentation comparison with external standards. Two characteristic ions were used for 7β-OH. Quantification was performed using calibration curves calculated with the internal standard. To control for the generation of 7β-OH during treatment of the samples, controls that contained only pure cholesterol (2.4 μmol/assay) were assayed in parallel. The amount of 7β-OH formed during treatment of the sample was below the detection limit of 0.02 nmol, indicating that <0.001% of the cholesterol was converted into 7β-OH during sample treatment.

Concentrations of individual tocopherols in plasma, liver, LDL, erythrocytes and dietary oils were determined by HPLC (21). Samples were mixed with 1 mL of 1% pyrogallol solution (in ethanol, absolute) and 150 μL of saturated sodium hydroxide solution. This mixture was heated for 30 min at 70°C, and tocopherols were extracted with n-hexane. Individual tocopherols of the extracts were separated isocratically using a mixture of n-hexane and 1,4 dioxane (96:4, v/v) as mobile phase and a LiChrosorb Si 60 column (5 μm particle size, 250 mm length, 4 mm i.d., Merck, Darmstadt, Germany) and detected by fluorescence (excitation wavelength: 295 nm; emission wavelength: 320 nm).

*Statistics.* Data were subjected to ANOVA including the factors fat and vitamin E and the interactions between fat and vitamin E using the Minitab Statistical Software (Minitab, State College, PA). When variances were heterogeneous, data were transformed into their logarithms before ANOVA. For statistically significant F-values, individual means of the treatment groups were compared by Fisher´s multiple range test. Means were considered significantly different at $P<0.05$.

# RESULTS

*Food intake and final body weights.* In rats fed coconut oil, food intake was independent of the dietary vitamin E concentration. In those fed salmon oil, intake of the rats whose diet contained 10 mg α-toc/kg was significantly lower than that of the groups whose diet contained 20, 40 or 240 mg α-toc/kg (**Table 1**). Daily body weight gains were affected by the interaction between the type of dietary fat and the dietary vitamin E supply. In rats fed coconut oil, daily body weight gains were independent of the dietary vitamin E concentration. In rats fed salmon oil, the rats whose diets contained 10 mg α-toc/kg had significantly lower body weight gains those whose diets contained 20, 40 or 240 mg α-toc/kg.

## TABLE 1

*Food intake, body weight gain and concentrations of total tocopherols in liver, plasma, LDL and erythrocytes of rats fed diets with coconut oil or salmon oil and four dietary vitamin E concentrations[1, 2]*

| | | Diet Vitamin E (mg α-tocopherol equivalents/kg) | | | |
| | Fat | 10 | 20 | 40 | 240 |
|---|---|---|---|---|---|
| Food intake,[*] *g/d* | Coconut oil | $17.6 \pm 0.9^{bc}$ | $18.1 \pm 0.6^{b}$ | $19.0 \pm 0.5^{ab}$ | $18.5 \pm 1.4^{ab}$ |
| | Salmon oil | $16.7 \pm 0.9^{c}$ | $17.8 \pm 0.6^{b}$ | $19.1 \pm 1.0^{a}$ | $18.3 \pm 1.7^{ab}$ |
| Body weight gain,[*†] *g/d* | Coconut oil | $7.0 \pm 0.5^{bc}$ | $7.0 \pm 0.5^{bc}$ | $7.3 \pm 0.7^{b}$ | $7.3 \pm 0.6^{b}$ |
| | Salmon oil | $6.5 \pm 0.4^{c}$ | $7.3 \pm 0.5^{b}$ | $8.1 \pm 0.9^{a}$ | $7.7 \pm 0.8^{ab}$ |
| | | Total tocopherols | | | |
| Liver,[#*†] *nmol/g* | Coconut oil | $26 \pm 4^{d}$ | $55 \pm 6^{c}$ | $67 \pm 13^{c}$ | $248 \pm 128^{a}$ |
| | Salmon oil | $1.0 \pm 0.3^{f}$ | $7.6 \pm 2.1^{e}$ | $29 \pm 9^{d}$ | $104 \pm 21^{b}$ |
| Plasma,[#*†] *μmol/L* | Coconut oil | $6.6 \pm 1.1^{d}$ | $13 \pm 2.9^{b}$ | $15 \pm 3.8^{b}$ | $21 \pm 5.1^{a}$ |
| | Salmon oil | $0.5 \pm 0.2^{g}$ | $1.7 \pm 0.5^{f}$ | $4.3 \pm 0.6^{e}$ | $8.5 \pm 1.5^{d}$ |
| LDL,[#*†] *μmol/L* | Coconut oil | $1.2 \pm 0.2^{c}$ | $2.0 \pm 0.5^{b}$ | $2.5 \pm 0.6^{ab}$ | $2.8 \pm 0.5^{a}$ |
| | Salmon oil | $0.2 \pm 0.1^{e}$ | $0.5 \pm 0.2^{d}$ | $1.2 \pm 0.4^{c}$ | $2.3 \pm 0.5^{ab}$ |
| Erythrocytes,[#*†] *nmol/g* | Coconut oil | $5.2 \pm 3.1^{c}$ | $6.7 \pm 1.8^{bc}$ | $11 \pm 5.9^{b}$ | $16 \pm 4.5^{a}$ |
| | Salmon oil | $0.7 \pm 0.3^{e}$ | $2.3 \pm 0.6^{d}$ | $5.1 \pm 1.7^{c}$ | $15 \pm 7.2^{ab}$ |

[1] Results are means ± SD, n=9.
[2] Values without a common superscript letter differ, *P<0.05*.
Significance of factors (*P<0.05*): [#] Fat, [*] vitamin E, [†] fat x vitamin E.

*Tocopherol concentrations.* In the rats fed diets containing 10 mg α-toc/kg, the concentrations of tocopherols in liver, plasma, LDL and erythrocytes were 5-26 times higher in those whose diet contained coconut oil rather than salmon oil (**Table 1**). Increasing the dietary vitamin E supply caused greater proportional increases in the tocopherol concentrations in all samples studied in rats fed salmon oil than in those fed coconut oil. Increasing the dietary vitamin E concentration from 10 to 240 mg α-toc/kg led to 12- to 100-fold increases in the tocopherol concentrations in liver, plasma, LDL and erythrocytes in rats fed salmon oil, whereas in rats fed coconut oil, the increases in tocopherol concentrations of these tissues were only 2- to 9-fold. At the highest vitamin E concentration of 240 mg α-toc/kg diet, the tocopherol concentrations of LDL and erythrocytes did not differ between rats whose diet contained salmon oil and those whose diet contained coconut oil. The tocopherol concentrations in liver and plasma, on the other hand, were more than twice as high in the rats whose diet contained coconut oil than in the rats whose diet contained salmon oil and a vitamin E concentration of 240 mg α-toc/kg.

*Concentrations of 7β-OH.* At the lowest vitamin E concentration, the rats whose diet contained salmon oil had 2.4-6 times higher concentrations of 7β-OH in the samples studied than those whose diet contained coconut oil (**Table 2**). In the rats fed salmon oil, increasing the vitamin E concentration from 10 mg α-toc/kg lowered the concentration of 7β-OH in all samples studied. The lowest concentrations of 7β-OH were found in liver and plasma of rats

fed diets comtaining vitamin E levels of 40 and 240 mg α-toc/kg and in LDL and erythrocytes in rats fed diets containing vitamin E levels of 20, 40 and 240 mg α-toc/kg. In rats fed coconut oil, the concentrations of 7β-OH in all samples studied did not differ due to vitamin E concentration. At vitamin E concentrations of 10 and 20 mg α-toc/kg, the rats fed salmon oil had higher concentrations of 7β-OH in all samples studied than those fed coconut oil. At vitamin E concentrations of 40 and 240 mg α-toc/kg, concentrations of 7β-OH differed between the rats fed coconut oil and salmon oil only in the liver, not in plasma, LDL or erythrocytes.

**TABLE 2**

*Concentrations of 7β-hydroxycholesterol in liver, plasma, LDL and erythrocytes of rats fed diets with coconut oil or salmon oil and four dietary vitamin E concentrations[1, 2]*

| Sample | Fat | Diet Vitamin E (mg α-tocopherol equivalents/kg) | | | |
|---|---|---|---|---|---|
| | | 10 | 20 | 40 | 240 |
| | | 7β-hydroxycholesterol | | | |
| Liver,[#*†] *nmol/g* | Coconut oil | $0.31 \pm 0.06^d$ | $0.43 \pm 0.11^d$ | $0.37 \pm 0.05^d$ | $0.44 \pm 0.04^d$ |
| | Salmon oil | $1.73 \pm 0.66^a$ | $1.36 \pm 0.42^b$ | $0.92 \pm 0.23^c$ | $1.06 \pm 0.31^{bc}$ |
| Plasma,[#*†] *nmol/L* | Coconut oil | $25 \pm 7^c$ | $22 \pm 3^c$ | $29 \pm 5^c$ | $35 \pm 5^c$ |
| | Salmon oil | $123 \pm 45^a$ | $58 \pm 8^b$ | $33 \pm 16^c$ | $34 \pm 11^c$ |
| LDL,[#*] *nmol/L* | Coconut oil | $20 \pm 5^{bc}$ | $14 \pm 6^{bc}$ | $16 \pm 4^{bc}$ | $11 \pm 3^c$ |
| | Salmon oil | $47 \pm 19^a$ | $26 \pm 5^b$ | $20 \pm 12^{bc}$ | $18 \pm 5^{bc}$ |
| Erythrocytes,[#*†] *nmol/g* | Coconut oil | $0.10 \pm 0.05^{bc}$ | $0.07 \pm 0.02^c$ | $0.09 \pm 0.02^{bc}$ | $0.10 \pm 0.02^{bc}$ |
| | Salmon oil | $0.26 \pm 0.08^a$ | $0.12 \pm 0.04^b$ | $0.11 \pm 0.03^{bc}$ | $0.10 \pm 0.03^{bc}$ |

[1] Results are means ± SD, n=9.
[2] Values without a common superscript letter differ, *P<0.05*.
Significance of factors (*P<0.05*): [#] Fat, [*] vitamin E, [†] fat x vitamin E.

**DISCUSSION**

In this study diets, containing coconut oil or salmon oil and different vitamin E concentrations were fed to rats. As expected, the vitamin E concentrations in the tissues were distinctly lower after feeding salmon oil than after feeding coconut oil. This is because PUFA, which are incorporated into membranes and lipoproteins, increase the use and depletion of tocopherols (22). The extremely low tocopherol concentrations in the tissues of rats whose diet contained the lowest vitamin E concentration and salmon oil suggest a deficiency of vitamin E, which would also explain their slower growth compared with the rats from all other groups.

Low vitamin E concentrations in tissues and LDL, combined with ingestion of highly unsaturated fatty acids as found in fish oil, markedly increased concentrations of 7β-OH. This is probably due to increased peroxidation of PUFA. High levels of highly unsaturated fatty acids and low tocopherol concentrations promote lipid peroxidation in tissues and lipoproteins (5, 6). The observation that the concentrations of 7β-OH in tissues were reduced by increasing the vitamin E supply confirms the importance of vitamin E as a protective factor against the formation of oxysterols. This is in agreement with several other studies that also suggested that the supply of antioxidants affects the formation of oxysterols (9-13). The study also shows very clearly, however, that an excessive supply of vitamin E does not further decrease the concentration of 7β-OH. The study indicates that even at a very high intake of highly unsaturated fatty acids, relatively moderate vitamin E concentrations of 20-40 mg α-tocopherol/kg diet are sufficient to protect the cholesterol in membranes and lipoproteins from oxidation.

The study also showed that the amount of vitamin E required to protect cholesterol from oxidation is distinctly lower in rats fed a fat with a low PUFA content than in rats fed a fat high in PUFA. At any given vitamin E supply, it is likely that fats low in PUFA lead to

higher vitamin E concentrations, lower concentrations of PUFA in tissues and lipoproteins and therefore a reduced susceptibility to oxidation than do fats high in PUFA (23).

Oxysterols in plasma and LDL are of particular pathophysiologic relevance. It has been shown that oxysterols as constituents of LDL are cytotoxic toward endothelial cells (24, 25). What is more, a correlation has been established between the concentration of 7β-OH in plasma and the etiology of arteriosclerosis (26, 27). The observation that at adequate vitamin E levels of 40 or 240 mg α-toc/kg diet, the concentrations of 7β-OH in plasma and LDL were not greater in rats fed salmon oil than in those fed coconut oil suggests that fish oil likely does not influence the formation of oxysterols when the supply with vitamin E is sufficient.

## LITERATURE CITED

1. Morel, D.W. & Lin, C.Y. (1996) Cellular biochemistry of oxysterols derived from the diet or oxidation in vivo. J. Nutr. Biochem. 7: 495-506.

2. Carpenter, K.L.H., Taylor, S.E., van der Veen, C., Williamson, B.K., Ballantine, J.A. & Mitchinson, M.J. (1995) Lipids and oxidised lipids in human atherosclerotic lesions at different stages of development. Biochim. Biophys. Acta 1256: 141-150.

3. Sevanian, A. & McLeod, L.L. (1987) Cholesterol Autoxidation in Phospholipid Membrane Bilayers. Lipids 22: 627-636.

4. Terao, J., Sugino, K. & Matsushita, S. (1985) $Fe^{2+}$ and Ascorbic Acid Induced Oxidation of Cholesterol in Phosphatidylcholine Liposomes and its Inhibition by alpha-Tocopherol. J. Nutr. Sci. Vitaminol. 31: 499-508.

5. Cho, S.-H. & Choi, Y. (1994) Lipid peroxidation and antioxidant status is affected by different vitamin E levels when feeding fish oil. Lipids 29: 47-52.

6. Hu, M.-L., Frankel, E.N., Leivovitz, D.E. & Tappel, A.L. (1989) Effect of dietary lipids and vitamin E on in vitro lipid peroxidation in rat liver and kidney homogenates. J, Nutr. 119: 1574-1582.

7. Kleinveld, H.A., Hak-Lemmers, H.L.M., Hectors, M.P.C., de Fouw, N., Demacker, P.N.M. & Stalenhoef, A.F.H. (1995) Vitamin E and fatty acid intervention does not attenuate the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbits. Arterioscler. Thromb. Vasc. Biol. 15: 290-297.

8. Brandsch, C., Ringseis, R. & Eder, K. (2002) High dietary iron concentrations enhance the formation of cholesterol oxidation products in the liver of adult rats fed salmon oil with minimal effects on antioxidant status. J. Nutr. 132: 2263-2269.

9. Mahfouz, M.M., Kawano, H. & Kummerow, F.A. (1997) Effect of Cholesterol-Rich Diets with and without Added Vitamins E and C on the Severity of Atherosclerosis in Rabbits. Am. J. Clin. Nutr. 66: 1240-1249.

10. Hodis, H.N., Chauhan, A., Hashimoto, S., Crawford, D.W. & Sevanian, A. (1992) Probucol Reduces Plasma and Aortic Wall Oxysterol Levels in Cholesterol Fed Rabbits Independently of its Plasma Cholesterol Lowering Effect. Atherosclerosis 96: 125-134.

11. Porkkala-Sarataho, E., Salonen, J.T., Nyyssönen, K., Kaikkonen, J., Salonen, R., Ristonmaa, U., Diczfalusy, U., Brigelius-Flohe, R., Loft, S. & Poulsen, H.E. (2000) Long-term effects of vitamin E, vitamin C, and combined supplementation on urinary 7-hydro-8-oxo-2′-deoxyguanosine, serum cholesterol oxidation products, and oxidation resistance of lipids in nondepleted men. Arterioscler. Thromb. Vasc. Biol. 20: 2087-2093.

12. Mol, M. J.T.M., Rijke de, Y., B., Demacker, P.N.M. & Stalenhoef, A.F.H. (1997) Plasma levels of lipid and cholesterol oxidation products and cytokines in diabetes mellitus and cigarette smoking: effects of vitamin E treatment. Atherosclerosis 129: 169-176.

13. Hodis, H.N., Hashimoto, S., Mack, W.J. & Sevanian A. (2000) Probucol reduces oxysterol formation in hypertensive rabbits. Hypertension 36: 436-441.

14. Adachi, J., Kudo, R., Ueno, Y., Hunter, R., Rajendram, R., Want, E. & Preedy, V.R. (2001) Heart 7-hydroperoxycholesterol and oxysterols are elevated in chronically ethanol-fed rats. J. Nutr. 131: 2916-2920.

15. Reeves, P.G., Nielsen, F.H. & Fahey, G.C. (1993) AIN-93 Purified Diets for Laboratory Rodents: Final Report of the American Institute of Nutrition ad hoc Writing Committee on the Reformulation of the AIN-76A Rodent Diet. J. Nutr. 123: 1939-1951.

16. Hara, A. & Radin, N.S. (1978) Lipid extraction of tissues with a low toxicity solvent. Anal. Biochem. 90, 420-426.

17. Association of Official Analytical Chemists (1980) Official Methods of Analysis (Horwitz, W., ed.), 13th ed., AOAC, Airlington, VA, pp. 440-441.

18. Butte, W. (1983) Rapid Method for the Determination of Fatty Acid Profiles from Fats and Oils using Trimethylsulfonium Hydroxide for Transesterification. J. Chromatogr. 261: 142-145.

19. Daenicke, S., Jeroch, H., Simon, O. & Bedford, M.R. (1999) Interactions Between Dietary Fat Type and Exogenous Enzyme Supplementation of Broiler Diets Based on Maize, Wheat, Triticale or Barley. J. Anim. Feed Sci. 8: 467-483.

20. Mori, T.A., Croft, K.D., Puddey, I.B. & Beilin, L.J. (1996) Analysis of Native and Oxidized Low-density Lipoprotein Oxysterols Using Gas Chromatography-Mass Spectrometry with Selective Ion Monitoring. Redox Report 2: 25-34.

21. Balz, M.K., Schulte, E. & Thier, H.P. (1993) Simultaneous Determination of Tocopherol Acetate, Tocopherols and Tocotrienols by HPLC with Fluorescence Detection in Foods. Fat Sci. Technol. 95: 215-220.

22. Farwer, S.R., Der Boer, B.C.J., Haddeman, E., Kivits, G.A.A., Wiersma, A. & Danse, B.H.J.C. (1994) The vitamin E nutritional status of rats fed on diets high in fish oil, linseed oil or sunflower seed oil. Br. J. Nutr. 72: 127-145.

23. Nardini, M., Scaccini, C., D´Aquino, M., Corcoss Benedetti, P., Di Felice, M. & Tomassi, G. (1993) Lipid peroxidation in liver microsomes of rats fed soybean, olive, and coconut oil. J. Nutr. Biochem. 4: 39-44.

24. Lizard, G., Monier, S., Cordelet, C., Gesquière, L., Deckert, V., Gueldry, S., Lagrost, L. & Gambert, P. (1999) Characterization and comparison of the mode of cell death, apoptosis versus necrosis, induced by 7β-hydroxycholesterol and 7-ketocholesterol in the cells of the vascular wall. Arterioscler. Thromb. Vasc. Biol. 19: 1190-1200.

25. Pirillo, A., Zhu, W., Roma, P., Galli, G., Caruso, D., Pellegatta, F. & Catalano, A.L. (1999) Oxysterols from oxidized LDL are cytotoxic but fail to induce hsp70 expression in endothelial cells. FEBS Lett. 462: 113-116.

26. Zieden, B. Kaminskas, A., Kristenson, M., Kucinskiene, Z., Vessby, B., Olsson, A.G. & Diczfalusy U. (1999) Increased plasma 7β-hydroxycholesterol concentrations in a population with a high risk for cardiovascular disease. Arterioscler. Thromb. Vasc. Biol. 19: 967-971.

27. Salonen, J.T., Nyyssönen, K., Salonen, R., Porkkala-Sarataho, E., Tuomainen T.-P., Diczfalusy U. & Björkhem, I. (1997) Lipoprotein oxidation and progression of carotid atherosclerosis. Circulation 95: 840-845.

## 3.4   Effekt oxidierten Cholesterins auf Parameter des antioxidativen Schutzsystems bei unterschiedlichen Diätfetten bei der Ratte

RINGSEIS R, EDER K: Effects of dietary oxidized cholesterol on the antioxidant defence system in rats fed coconut oil or salmon oil. *International Journal for Vitamin and Nutrition Research* (Manuskript: eingereicht zur Publikation 2002)

**Summary:** An experiment was conducted with rats to investigate the effect of dietary oxidized cholesterol on the antioxidant status. Four groups of male, growing Sprague-Dawley rats received diets containing pure or oxidized cholesterol (5 g/kg diet) with either coconut oil or salmon oil as dietary fat (100 g/kg diet) for 5 weeks. Rats fed diets containing oxidized cholesterol had significantly higher concentrations of 7β-hydroxycholesterol and 7-ketocholesterol in the liver than rats fed diets containing pure cholesterol. Moreover, rats fed the diets containing oxidized cholesterol had significantly higher mRNA concentrations of glutathione peroxidase and superoxide dismutase, a significantly higher activity of glutathione peroxidase and significantly lower concentrations of total and reduced glutathione in the liver than rats fed diets containing pure cholesterol. These effects were independent of the dietary fat. The study suggests that dietary oxidized cholesterol stresses the antioxidant defence system in rats.

**Introduction**

Numerous studies indicate that oxysterols, being constituents of LDL, are involved in the pathogenesis of atherosclerosis [1, 2]. Oxysterols are formed not only endogenously by enzymatic or non-enzymatic processes, but also enter the circulation and the liver as a result of consuming cholesterol-rich foods [3, 4]. In vitro studies in human macrophages and monocytes and in rat hepatocytes showed that oxysterols such as 7β-hydroxycholesterol (7β-OH) and 7-ketocholesterol (7-K) have cytotoxic properties and induce oxidant stress which manifests itself by glutathione (GSH) depletion and increased activity of antioxidant enzymes [5-8]. There are, however, no studies investigating the potential effect of oxysterols on the antioxidant status in vivo. The purpose of the present study was therefore to ascertain whether dietary oxysterols affect the antioxidant defence system in the liver.

It is known that the type of dietary fat also affects the antioxidant status. Fats with a high proportion of polyunsaturated fatty acids (PUFA) like fish oil for example stress the antioxidant defence system because of the high susceptibility of these fatty acids to oxidation. It is therefore reasonable to assume that the effect of oxidized cholesterol on the antioxidant defence system will also depend on the type of dietary fat. We expected that the effects of oxidized cholesterol on the antioxidant defence system would be magnified if a fat with a high proportion of PUFA was fed at the same time. Studies on potential interactions between oxysterols and the type of dietary fat are lacking so far.

In order to consider the effect of oxysterols in isolation, we selected a study design where the effect of an oxidized cholesterol (oxChol) preparation was compared with untreated cholesterol. In order to simulate a practical human diet we used an oxChol preparation that was only moderately oxidized. Most other studies investigating the effects of oxysterols used oxChol preparations that were almost completely oxidized [9, 10]. But in an average Western mixed diet only about 1% of the ingested cholesterol is present in oxidized form [11]. Rats were the model objects in our study. The concentrations of tocopherols and GSH in the liver and the gene expression and activities of several antioxidant enzymes in the liver were determined as parameters for the antioxidant defence system. We know from the literature that these parameters reflect the antioxidant status and expose nutritive oxidant stress [12-15].

**Materials and methods**

*Animals:* 36 male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) with an initial body weight of 72 g (± 7 g, SD) were assigned to four groups of nine rats each. The animals were kept individually in Macrolon cages in a room maintained at a temperature of 23° C and 50 to 60% relative humidity with lighting from 0700 to 1900 h. All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt.

*Diets and feeding:* Semisynthetic diets were used. The basal diet consisted of (g/kg diet): corn starch (398), casein (200), saccharose (200), fat (100), cellulose (35), vitamins and minerals (60) and DL-methionine (2). Minerals and vitamins with the exception of vitamin E were supplemented in accordance with recommendations by the American Institute of Nutrition AIN-93G [16] for rat diets. The dietary fat (coconut oil vs. salmon oil) and cholesterol obtained from Sigma-Aldrich (Steinheim, Germany) (5 g pure cholesterol per kg vs. 5 g oxidized cholesterol per kg) were varied according to a two-factorial design. Oxidized cholesterol was prepared by heating cholesterol (Sigma-Aldrich, Steinheim, Germany) placed as a thin film on a glass Petri dish at 115°C for 48 hours in an electric oven.

To equalize the vitamin E concentrations of the diets irrespective of the dietary fats used, the native tocopherol concentrations of the two fats were analysed. Based on these native concentrations, diets were supplemented individually with all-*rac*-α-tocopheryl acetate (Merck Eurolab, Darmstadt, Germany), allowing for a biopotency of 67% compared to α-tocopherol. After supplementation the vitamin E concentrations of both types of diets were 40 mg α-tocopherol equivalents/kg. The fatty acid composition of coconut oil (Palmin, Hamburg, Germany) and salmon oil (Caelo, Hilden, Germany) is shown in *Table I*.

*Table I:* Fatty acid composition of the dietary fats (g/100g fatty acids)

| Fatty acid | Coconut oil | Salmon oil |
|---|---|---|
| Caprylic acid (8:0) | 8.1 | -[*] |
| Capric acid (10:0) | 6.3 | -[*] |
| Lauric acid (12:0) | 45.6 | 0.1 |
| Myristic acid (14:0) | 17.0 | 6.1 |
| Palmitic acid (16:0) | 9.7 | 14.5 |
| Palmitoleic acid (16:1) | -[*] | 8.2 |
| Hexadecadienoic acid (16:2) | -[*] | 1.4 |
| Hexadecatetraenoic acid (16:4) | -[*] | 1.4 |
| Stearic acid (18:0) | 4.5 | 3.0 |
| Oleic acid (18:1 n-9) | 6.3 | 12.9 |
| Octadecenoic acid (18:1 n-7) | -[*] | 2.7 |
| Linoleic acid (18:2) | 1.9 | 2.2 |
| α-Linolenic acid (18:3 n-3) | 0.08 | 0.8 |
| Eicosanoic acid (20:1) | 0.05 | 4.3 |
| Eicosatrienoic acid (20:3 n-6) | -[*] | 0.3 |
| Arachidonic acid (20:4 n-6) | -[*] | 0.8 |
| Eicosatetraenoic acid (20:4 n-3) | -[*] | 1.4 |
| Eicosapentaenoic acid (20:5 n-3) | -[*] | 12.7 |
| Docosanoic acid (22:1) | -[*] | 4.5 |
| Docosapentaenoic acid (22:5 n-3) | -[*] | 3.1 |
| Docosahexaenoic acid (22:6 n-3) | -[*] | 10.0 |

[*] - not detected (<0.05g/100g fatty acids)

The peroxide values of the dietary fats, which were extracted from the diets with a mixture of hexane and isopropanol (3:2, as described in [17]) and measured according to official methods [18], were 3.9 and <0.1 mEq $O_2$ per kg salmon oil and coconut oil, respectively.

The diets were prepared weekly by solubilizing the all-*rac*-α-tocopheryl acetate and pure cholesterol/oxidized cholesterol preparation in the fat and mixing with the dry

components and water. The diets were then freeze dried and stored at –20°C to prevent autoxidation of lipids, e.g. polyunsaturated fatty acids and cholesterol. The water content after freeze drying was below 5 g per 100 g of diet.

To standardize the feed intake, the diets were fed daily in restricted amounts at 0800 h. The feed intake was increased from 7.0 g per day to 15.0 g per day during the experiment resulting in an average daily feed intake of 14.4 g. Water was provided ad libitum from nipple drinkers. The experimental diets were fed for 35 days.

*Sample collection:* After 35 days of feeding the experimental diets the rats were starved overnight, anesthetized with diethyl ether and killed by decapitation. The liver was excised immediately, frozen with liquid nitrogen and stored at -80°C pending analysis.

*Lipid analysis:* The fatty acid composition of the experimental fats was determined by gas chromatography of fatty acid methyl esters (FAMEs) [19]. FAMEs were separated in a HP 5890 gas chromatographic system (Hewlett-Packard, Taufkirchen, Germany) fitted with a polar capillary column (FFAP, 30 m, 0.53 mm internal diameter, Machery and Nagel, Düren, Germany), detected by flame ionization and identified by comparing their retention times with those of individually purified standards [20].

*Oxysterols:* 7β-OH and 7-K in the liver and oxysterols (Sigma-Aldrich, Steinheim, Germany) in the diets were determined using a quantitative GC-MS method with selective ion monitoring [21], which has been described in detail in a previous paper [22].

*Antioxidant status:* Concentrations of individual tocopherols in the liver and dietary fats were determined using a HPLC method [23].

The activity of glutathione peroxidase (GSH-Px) in the liver was determined with t-butyl hydroperoxide at 25°C according to the method of Paglia and Valentine [24]. One unit of GSH-Px activity is defined as one μmol reduced β-nicotinamide adenine dinucleotide phosphate oxidized per min. Determination of total ($GSH_{tot}$) and oxidized glutathione (GSSG) was performed in protein-free liver homogenates according to Tietze [25] with glutathione reductase and Ellman´s reagent. Reduced glutathione ($GSH_{red}$) was calculated by subtracting two times the GSSG value from the $GSH_{tot}$ value. Calibration was performed using a standard curve. The activity of glucose-6-phosphate dehydrogenase (G6PDH) in the liver was determined by the method of Deutsch [26]. One unit of G6PDH is defined as one μmol reduced β-nicotinamide adenine dinucleotide phosphate oxidized per min.

*Measurement of relative mRNA concentrations:* To quantify the gene expression of GSH-Px, SOD and GSH reductase the total RNA was isolated from liver by Trizol[TM] reagent (Invitrogen, Karlsruhe, Germany). The relative quantity of GSH-Px mRNA compared to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA was determined by means of semiquantitative RT-PCR using a commercially available one step RT-PCR kit (Amersham Pharmacia Biotech, Freiburg, Germany) and specific primers coding for GSH-Px and GAPDH. The PCR mixtures were subjected to 30 cycles of amplification at 95°C 30 s, 60°C 30 s and 72°C 45 s. The PCR products were then separated by ethidium bromide-containing agarose gel electrophoresis, digitalized with a digital camera (SynGene, Cambridge, England) using SynGene-Software (GeneSnap and GeneTools) for identification and quantification. The relative quantities of SOD mRNA and GSH reductase mRNA compared to GAPDH mRNA were determined by quantitative real-time RT-PCR. First strand cDNA was prepared using the Omniscript RT Kit (200) from QIAGEN (Hilden, Germany) and Oligo dT-Primer pd(T)$_{12-18}$ from Amersham Pharmacia Biotech (Freiburg, Germany). The real-time PCR was performed with a Taq DNA Polymerase from Promega (Mannheim, Germany) and specific primers coding for GAPDH, SOD and GSH reductase. 35 Cycles of amplification (95°C 20 s, 60°C 30 s and 72°C 40 s) were performed using a real-time RCR cycler (Rotorgene, Corbett Research, LTF Labortechnik, Wasserburg, Germany). The primers used for PCR were as follows: 5´-GCATGGCCTTCCGTGTTCC-3´ (forward) and 5´-GGGTGGTCCAGGGTTTCTTACTC-3´ (reverse) for rat GAPDH; 5´-

CACCACGACCCGGGACTACACC-3´ (forward) and 5´-CACCAAGCCCAGATACCAGGAATG-3´ (reverse) for rat GSH-Px; 5´-TCCGGTGCAGGGCGTCATTC-3´ (forward) and 5´-ACACATTGGCCACACCGTCCTT-3´ (reverse) for rat SOD; 5´-ACGCTGGCGGTGTTGAGGTTCT-3´ (forward) and 5´-TGAGCCCCACTGTCCCGATAGG-3´ (reverse) for rat GSH reductase.

*Statistics:* Data were subjected to ANOVA using the Minitab Statistical Software (Minitab, State College, PA, USA). Classification factors were dietary fat, cholesterol and the interaction of both factors. In cases where the differences between variances and means were large, data were transformed to logarithms prior to ANOVA. For statistically significant F values, individual means of the treatment groups were compared by Fisher´s multiple range test. Means were considered significantly different for $P<0.05$.

## Results

*Concentrations of oxysterols in the diets:* The diets supplemented with 5 g oxidized cholesterol/kg contained the following oxysterols (mg/kg) which are known to be formed at the highest concentrations during heating of cholesterol: 7β-OH, 2.7; α-epoxycholesterol, 2.6; β-epoxycholesterol, 6.9; cholestanetriol, 0.1; 25-hydroxycholesterol, 9.4; 7-ketocholesterol, 1.7. The concentration of cholesterol was 4.63 g/kg diet in the diet supplemented with oxidized cholesterol. Other minor, unidentified oxysterols have not been quanified. In the diets containing pure cholesterol the concentrations of all oxysterols were below the limit of detection of 0.025 mg/kg.

*Food intake and final body weight of the rats.* The final body weight of the rats was significantly affected by the type of dietary fat. Rats fed the salmon oil diets had significantly higher final body weights than rats fed the coconut oil diets (salmon oil: $284 \pm 10$ g; coconut oil: $263 \pm 11$ g, means $\pm$ SD, n=18 for each group, $P<0.05$). The type of cholesterol (oxidized vs. pure cholesterol) did not affect the rats' final body weight.

*Concentrations of 7β-OH and 7-K in the liver (Table II).* The concentrations of 7β-OH and 7-K were affected by the dietary fat and the type of cholesterol. Rats fed the salmon oil diets had 3.3 times higher concentrations of 7ß-OH and 3.5 times higher concentrations of 7-K in the liver than rats fed the coconut oil diets. Rats fed the diets containing oxidized cholesterol had 1.7 higher concentrations of 7ß-OH and 2.3 times higher concentrations of 7-K than rats fed the diets containing pure cholesterol. The concentrations of both oxysterols were not affected by interactions between dietary fat and type of cholesterol.

*Antioxidant status of the liver (Table II).* The concentration of α-tocopherol in the liver was not different between rats fed diets containing pure cholesterol and rats fed diets containing oxidized cholesterol. However, rats whose diet contained coconut oil had significantly higher concentrations of α-tocopherol than rats whose diets contained salmon oil. Rats fed diets containing oxidized cholesterol had a significantly higher activity of GSH-Px than rats fed diets containing pure cholesterol. Rats fed the diets containing oxidized cholesterol, moreover, had significantly lower concentrations of total and reduced glutathione in the liver than rats fed the diets containing pure cholesterol. The activity of G6PDH was markedly lower in rats fed salmon oil diets than in rats fed coconut oil diets but was not affected by the type of cholesterol. The activities of antioxidant enzymes and concentrations of total and reduced glutathione were not affected by interactions between dietary fat and type of cholesterol.

*Table II:* Concentrations of 7β-hydroxycholesterol and 7-ketocholesterol, α-tocopherol and total and reduced glutathione and activities of antioxidant enzymes in the liver of rats fed a diet containing 0.5% cholesterol or 0.5% oxidized cholesterol at two different dietary fats (coconut oil vs. salmon oil)[1,2]

| Fat | Coconut oil | | Salmon oil | |
|---|---|---|---|---|
| Cholesterol | Cholesterol | oxidized Cholesterol | Cholesterol | oxidized Cholesterol |
| 7β-hydroxycholesterol (nmol/g) [3,4] | $6.5 \pm 1.9^d$ | $13.6 \pm 1.9^c$ | $25.7 \pm 3.7^b$ | $40.2 \pm 13.0^a$ |
| 7-ketocholesterol (nmol/g) [3,4] | $24.1 \pm 6.1^d$ | $112 \pm 38^c$ | $164 \pm 43^b$ | $312 \pm 60^a$ |
| α-tocopherol (nmol/g) [3] | $138 \pm 35^a$ | $149 \pm 28^a$ | $96 \pm 21^b$ | $83 \pm 16^b$ |
| GSH-Px (U/mg protein) [4] | $1.02 \pm 0.13^b$ | $1.39 \pm 0.17^a$ | $1.15 \pm 0.20^b$ | $1.43 \pm 0.17^a$ |
| G6PDH (U/mg protein) [3] | $6.94 \pm 2.38^a$ | $8.21 \pm 3.93^a$ | $1.07 \pm 0.24^b$ | $1.22 \pm 0.28^b$ |
| GSH$_{tot}$ (μmol/g) [4] | $3.15 \pm 0.50^{ab}$ | $2.75 \pm 0.50^b$ | $3.24 \pm 0.43^a$ | $2.99 \pm 0.38^{ab}$ |
| GSH$_{red}$ (μmol/g) [4] | $2.20 \pm 0.32^{ab}$ | $1.87 \pm 0.18^b$ | $2.36 \pm 0.31^a$ | $2.02 \pm 0.30^b$ |

[1]Results are means ± SD, n=9 for each treatment group. Means within a row not sharing the same superscript letters differ significantly by Fisher´s multiple range test (P<0.05).
[2]Significance of Factors (P<0.05): [3]Fat, [4]Cholesterol, [5]Fat x cholesterol.

*mRNA concentration of antioxidant enzymes (Table III).* Rats fed diets containing oxidized cholesterol had significantly higher mRNA concentrations of GSH-Px and SOD than rats fed diets containing pure cholesterol. Rats fed the diets containing oxidized cholesterol also showed a tendency (P<0.15) towards higher mRNA concentrations of GSH reductase as compared to rats fed diets with pure cholesterol. The concentrations of mRNA of all the enzymes studied were not affected by the dietary fat and no interactions occurred between dietary fat and type of cholesterol.

*Table III:* Relative mRNA concentrations (% of GAPDH) of antioxidative enzymes in the liver of rats fed a diet containing 0.5% cholesterol or 0.5% oxidized cholesterol at two different dietary fats (coconut oil vs. salmon oil)[1,2]

| Fat | Coconut oil | | Salmon oil | |
|---|---|---|---|---|
| Cholesterol | Cholesterol | oxidized Cholesterol | Cholesterol | oxidized Cholesterol |
| | | (% of GAPDH) | | |
| mRNA GSH-Px [3] | $206 \pm 20.1^b$ | $286 \pm 14.1^a$ | $225 \pm 48.7^b$ | $298 \pm 41.9^a$ |
| mRNA SOD [3] | $30.4 \pm 4.41^b$ | $38.8 \pm 9.77^a$ | $32.6 \pm 4.39^{ab}$ | $38.0 \pm 6.10^a$ |
| mRNA GSH reductase | $0.44 \pm 0.18^b$ | $0.53 \pm 0.18^{ab}$ | $0.53 \pm 0.18^{ab}$ | $0.68 \pm 0.21^a$ |

[1]Results are means ± SD, n=9 for each treatment group. Means within a row not sharing the same superscript letters differ significantly by Fisher´s multiple range test (P<0.05).
[2]Significance of Factors (P<0.05): [3]Cholesterol.

**Discussion**

In this study we investigated whether dietary oxysterols affect the antioxidant status of rats. The experimental animals were fed a restrictive diet in order to exclude secondary effects which might result from different feed intakes. Yet to our surprise, the rats whose diet contained salmon oil had higher body weights at the end of the experiment than the rats

whose diet contained coconut oil. This might be due to the low concentrations of linoleic and α-linolenic acid in the coconut oil diets, although raised concentrations of 20:3 (n-9) in liver lipids, which would indicate a deficiency of essential fatty acids, were not observed (data not shown). The differences in body weights between the rats are however unlikely to jeopardize the results of our study with regard to the effects of oxidized cholesterol.

As expected, the consumption of oxidized cholesterol vs. pure cholesterol lead to increased concentrations of 7β-OH and 7-K in the liver. This is due to the fact that oxysterols are absorbed from the diet and carried to the liver where they are partially incorporated [3, 4]. The observation that rats whose diet contained fish oil had higher concentrations of 7β-OH and 7-K in the liver than rats whose diet contained coconut oil is probably due to the fact that incorporation of highly unsaturated fatty acids into liver lipids makes them much more prone to oxidation. This is consistent with the assumption that 7β-OH and 7-K, unlike other oxysterols, are secondary products of peroxidation of PUFA [27]. Other studies have also shown that oxidation of PUFA can secondarily lead to oxidation of cholesterol [22, 28].

The study also shows that consumption of oxidized cholesterol as opposed to pure cholesterol leads to increased expression and activity of antioxidant enzymes in the liver and a reduced concentration of glutathione. Induction of the expression of antioxidant enzymes and a reduced concentration of glutathione in the liver have both been described as a consequence of oxidant stress [15, 29]. The present study also suggests that consumption of oxidized cholesterol as opposed to pure cholesterol leads to oxidant stress in the liver. It was noted that the effects of oxidized cholesterol on expression and activity of the named enzymes were as strong after feeding coconut oil as after feeding fish oil. This shows that oxidized cholesterol stresses the antioxidant defence system even if the susceptibility of tissues to oxidation is low. The present study in rats is therefore consistent with studies in cell systems, where oxysterols such as 7β-OH and 7-K also induced oxidant stress. Oxysterols led to increased activities of antioxidant enzymes and depletion of glutathione also in human macrophages and monocytes and in rat hepatocytes [5-8]. In studies with monocytes or macrophages the depletion of glutathione through oxysterols was described as having great pathophysiological significance [5, 30, 31].

The study also shows that the vitamin E status of the liver was not significantly affected by oxidized cholesterol, irrespective of the type of dietary fat. This suggests that oxidized cholesterol, unlike oxidized fatty acids [32], does not raise the vitamin E consumption in tissues, which leads us to conclude that ingestion of oxidized cholesterol does not significantly increase the requirement for vitamin E.

To summarise, the results of this study support the conclusion that consumption of oxidized cholesterol causes oxidant stress in the liver of rats. We can therefore assume that the prooxidant effects described probably contribute to the pathophysiological effects of oxysterols.

## References

1.  Colles, S.M., Maxson, J.M., Carlson, S.G. and Chisolm, G.M. (2001) Oxidized LDL-induced injury and apoptosis in atherosclerosis. Potential roles for oxysterols. Trens Cardiovasc. Med. *11*, 131-138.
2.  Carpenter, K.L.H., Taylor, S.E., Ballantine, J.A., Fussell, B., Halliwell, B. and Mitchinson, M.J. (1993) Lipids and oxidized lipids in human atheroma and normal aorta. Biochim. Biophys. Acta *1167*, 121-130.
3.  Linseisen, J. and Wolfram, G. (1998) Absorption of cholesterol oxidation products from ordinary foodstuffs in humans. Ann. Nutr. Metab. *42*, 221-230.
4.  Vine, D.F., Croft, K.D., Beilin, L.J. and Mamo, J.C.L. (1997) Absorption of dietary cholesterol oxidation products and incorporation into rat lymph chylomicrons. Lipids *32*, 887-893.
5.  O´Callaghan, Y.C., Woods, J.A. and O´Brien, N.M. (2002) Characteristics of 7β-hydroxycholesterol-induced cell death in a human monocytic blood cell line, U937, and a human hepatoma cell line, HepG2. Toxicol. in Vitro *16*, 245-251.

6. Bansal, G., Singh, U. and Bansal, M.P. (2001) Effect of 7β-hydroxycholesterol on cellular redox status and heat shock protein 70 expression in macrophages. Cell Physiol. Biochem. *11*, 241-246.

7. Lyons, N.M., Woods, J.A. and O´Brien, N.M. (2001) α-Tocopherol, but not γ-Tocopherol inhibits 7β-hydroxycholesterol-induced apoptosis in human U937 cells. Free Rad. Res. *35*, 329-339.

8. Cantwell, H. and Devery, R. (1998) The response of the antioxidant defense system in rat hepatocytes challenged with oxysterols is modified by Covi-ox. Cell Biol. Toxicol. *14*, 401-409.

9. Osada, K., Kodama, T., Yamada, K., Nakamura, S. and Sugano, M. (1998) Dietary oxidized cholesterol modulates cholesterol metabolism and linoleic acid desaturation in rats fed high-cholesterol diets. Lipids *33*, 757-764.

10. Osada, K., Kodama, T., Cui, L., Ito, Y. and Sugano, M. (1994) Effects of dietary oxidized cholesterol on lipid metabolism in differently aged rats. Biosci. Biotech. Biochem. *58*, 1062-1069.

11. Bovenkamp van de, P., Kosmeijer-Schuil, T.G. and Katan, M.B. (1988) Quantification of oxysterols in dutch foods: egg products and mixed diets. Lipids *23*, 1079-1085.

12. Hu, M.-L., Frankel, E.N., Leibovitz, B.E. and Tappel, A.L. (1989) Effect of dietary lipids and vitamin E on in vitro lipid peroxidation in rat liver and kidney homogenates. J. Nutr. *119*, 1574-1582.

13. Ruiz-Gutierrez, V., Perez-Espirosa, A., Vazquez, C.M. and Santa-Maria, C. (1999) Effects of dietary fats (fish, olive and high-oleic-acid sunflower oils) on lipid composition and antioxidant enzymes in rat liver. Br. J. Nutr. *82*, 233-241.

14. Chen, L.-C., Boissonneault, G., Hayek, M.G. and Chow, C.K. (1993) Dietary fat effects on hepatic lipid peroxidation and enzymes of H2O2 metabolism and NADPH generation. Lipids *28*, 657-662.

15. Shull, S., Heintz, N.H., Periasamy, M., Manohar, M., Janssen, Y.M., Marsh, J.P. and Mossman, B.T. (1991) Differential regulation of antioxidant enzymes in response to oxidants. J. Biol. Chem. *266*, 24398-24403.

16. Reeves, P.G., Nielsen, F.H. and Fahey, G.C. (1993) AIN-93 purified diets for laboratory rodents: Final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN 76A rodent diet. J. Nutr. *123*, 1939-1951.

17. Hara, A. and Radin, N.S. (1978) Lipid extraction of tissues with a low toxicity solvent. Anal. Biochem. *90*, 420-426.

18. Association of Official Analytical Chemists (1980) Official Methods of Analysis (Horwitz, W., ed.), 13th ed., pp. 440-441, AOAC, Airlington.

19. Butte, W. (1983) Rapid method for the determination of fatty acid profiles from fats and oils using trimethylsulfoniumhydroxide for transesterification. J. Chromatogr. *261*, 142-145.

20. Daenicke, S., Jeroch, H., Simon, O. and Bedford, M.R. (1999) Interactions between dietary fat type and exogenous enzyme supplementation of broiler diets based on maize, wheat, triticale or barley. J. Anim. Feed Sci. *8*, 467-483.

21. Mori, T.A., Croft, K.D., Puddey, I.B. and Beilin, L.J. (1996) Analysis of native and oxidized low-density lioprotein oxysterols using gas chromatography-mass spectrometry with selective ion monitoring. Redox Report *2*, 25-34.

22. Brandsch, C., Ringseis, R. and Eder, K. (2002) High dietary iron concentrations enhance the formation of cholesterol oxidation products in the liver of adult rats fed salmon oil with minimal effects on the antioxidant status. J. Nutr. *132*, 2263-2269.

23. Balz, M.K., Schulte, E. and Their, H.P. (1993) Simultaneous determination of tocopherol acetate, tocopherols and tocotrienols by HPLC with fluorescence detection in foods. Fat Sci. Technol. *95*, 215-220.

24. Paglia, D.E. and Valentine, W.N. (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. *70*, 158-169.

25. Tietze, F. (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal. Biochem. *27*, 502-522.

26. Deutsch, J. (1985) Glucose-6-Phosphat-Dehydrogenase. In: Methods of enzymatic analysis (Bergmeyer, H.U., ed.) vol. 3, pp. 190-197, VCH Verlagsgesellschaft mbH, Weinheim.

27. Björkhem, I. (2002) Do oxysterols control cholesterol homeostasis? J. Clin. Invest. *110*, 725-730.

28. Ringseis, R. and Eder, K. (2002) Insufficient dietary vitamin E increases 7β-hydroxycholesterol in tissues of rats fed salmon oil. J. Nutr. *132*, 3732-3735.

29. Ibrahim, W., Lee, U.-S., Yeh, C.-C., Szabo, J., Bruckner, G. and Chow, C.K. (1997) Oxidative stress and antioxidant status in mouse liver: effects of dietary lipid, vitamin E and iron. J. Nutr. *127*, 1401-1406.

30. Lizard, G., Gueldry, S., Sordet, O., Monier, S., Athias, A., Miguet, C., Bessede, G., Lemaire, S., Solary, E. and Gambert, P. (1998) Glutathione is implied in the control of 7-ketocholesterol-induced apoptosis, which is associated with radical oxygen species production. FASEB J. *12*, 1651-1663.

31. Lizard, G., Lemaire, S., Monier, S., Gueldry, S., Néel, D. and Gambert, P. (1997) Induction of apoptosis and of interleukin-1ß secretion by 7β-hydroxycholesterol and 7-ketocholesterol: partial inhibition by Bcl-2 overexpression. FEBS Lett. *419*, 276-280.

32. Eder, K. (1999) The effects of a dietary oxidized oil on lipid metabolism in rats. Lipids *34*, 717-725.

**3.5    Effekt oxidierten Cholesterins auf Parameter des Lipidstoffwechsels und der VLDL-Synthese und -Sekretion bei unterschiedlichen Diätfetten bei der Ratte**

**ABSTRACT**  The aim of this study was to compare the effects of dietary oxidized cholesterol and pure cholesterol on plasma and VLDL lipids and on some parameters of VLDL assembly and secretion in rats fed two different dietary fats. Four groups of male growing Sprague-Dawley rats were fed diets containing pure or oxidized cholesterol (5 g/kg diet) with either coconut oil or salmon oil as dietary fat (100 g/kg diet) for 35 days. Rats fed oxidized cholesterol supplemented diets had significantly lower concentrations of triglycerides and cholesterol in plasma and VLDL than rats fed pure cholesterol supplemented diets irrespective of the type of fat. In addition, rats fed oxidized cholesterol supplemented diets had significantly lower relative concentrations of microsomal triglyceride transfer protein mRNA than rats fed pure cholesterol supplemented diets. In contrast, hepatic lipid concentrations and the relative concentration of apolipoprotein B mRNA were not influenced by the dietary factors investigated. Parameters of hepatic lipogenesis (relative mRNA concentration of sterol regulatory element binding protein-1c and activity of glucose-6-phosphat dehydrogenase) were significantly reduced by feeding fish oil compared to coconut oil, but were not affected by the type of cholesterol. In conclusion, the data of this study suggest, that dietary oxidized cholesterol affects VLDL assembly and/or secretion by reducing the synthesis of MTP but not by impairing hepatic lipogenesis or synthesis of apolipoprotein B.

**Introduction**
Results from animal studies exhibited that dietary oxidized cholesterol influences lipid metabolism [1-3]. Several studies showed that dietary oxidized cholesterol leads to decreased concentrations of triglycerides and/or cholesterol in plasma as compared to pure cholesterol [3-5].

Plasma lipids mainly derive from the liver, which are secreted by the liver within apolipoprotein B (apoB) containing lipoproteins (very low density lipoprotein, VLDL). Dietary cholesterol enhances VLDL synthesis and secretion through an increase in the transcription of microsomal triglycerid transfer protein (MTP) [6]. MTP is essential for the assembly of apoB containing lipoproteins by translocation of apoB and component lipids (cholesterol, cholesterol esters, triglycerides, and phospholipids) across the endoplasmatic reticulum [7]. Transcription of the MTP gene is regulated by cholesterol through a sterol response element located in its promoter [8]. Specific oxysterols are potent regulators of lipid metabolism through activation of liver X receptor α (LXRα), a nuclear transcription factor involved in the regulation of genes important for cholesterol homeostasis [9]. Therefore, it appears to be possible that dietary oxidized cholesterol alters the synthesis and secretion of VLDL by affecting the transcription of MTP and/or apoB, too. However, the effects of dietary oxidized cholesterol on VLDL synthesis or secretion have not yet been investigated. Hence, this study was conducted to determine the effects of oxidized cholesterol on plasma and VLDL lipids and the gene expression of apoB and MTP in rats. Rats fed pure cholesterol were used as controls.

Concentrations of VLDL lipids are strongly influenced by hepatic lipid concentrations, too. Concerning, that dietary oxidized cholesterol was shown to affect hepatic cholesterol biosynthesis and lipogenesis [1, 2, 5], we proposed to measure the concentrations of

cholesterol, triglycerides and phospholipids in the liver. As parameters of hepatic lipogenesis we determined the gene expression of the sterol regulatory element binding protein-1c (SREBP-1c) and the activity of the lipogenic enzyme glucose-6-phosphate dehydrogenase (G-6-PDH). SREBP-1c, a target gene of LXRα, is a transcription factor that regulates expression of various lipogenic genes. Therefore, SREBP-1c not only coordinates the regulation of hepatic lipogenesis but also controls the assembly and secretion of apoB-containing lipoproteins [10]. As the type of fat shows a marked influence on hepatic lipid synthesis and plasma lipid concentrations we planned to use two dietary fats, salmon oil and coconut oil, with a very different fatty acid composition. Fish oil shows a suppressive effect on hepatic lipogenesis, which is mediated by a reduced transcription of lipogenic enzymes, which are controlled by SREBP-1c [11]. Dietary oxidized cholesterol was shown to modulate desaturation of fatty acids within liver phospholipids [1, 2, 12], which might influence the concentration of VLDL component lipids. Therefore, we further determined the fatty acid composition of hepatic phospholipids and triglycerides to assess possible effects of oxidized cholesterol on the desaturation of fatty acids.

## Materials and methods

### Animals and diets

36 male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) with an initial body weight of 72 g (± 7 g, SD) were assigned to four groups of nine rats each. The animals were kept individually in Macrolon cages in a room maintained at a temperature of 23° C and 50 to 60% relative humidity with lighting from 0700 to 1900 h. All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt.

Semisynthetic diets were used. The composition of the basal diets is shown in Table 1. Minerals and vitamins with the exception of vitamin E were supplemented in accordance with recommendations by the American Institute of Nutrition AIN-93G [13] for rat diets. The dietary fat (coconut oil vs. salmon oil) and cholesterol obtained from Sigma-Aldrich (Steinheim, Germany) (5 g pure cholesterol per kg vs. 5 g oxidized cholesterol per kg) were varied according to a two-factorial design. Oxidized cholesterol was prepared by heating cholesterol (Sigma-Aldrich, Steinheim, Germany) placed as a thin film on a glass Petri dish at 115°C for 48 hours in an electric oven. Oxysterols (Sigma-Aldrich, Steinheim, Germany) in the diets were determined using a quantitative GC-MS method with selective ion monitoring [14], which has been described in detail in a previous paper [15].

**Table 1**        Composition of the basal diets

| Ingredient | Cholesterol (g/kg diet) | Oxidized Cholesterol (g/kg diet) |
|---|---|---|
| Corn starch | 398 | 398 |
| Casein | 200 | 200 |
| Saccharose | 200 | 200 |
| Fat[*] | 100 | 100 |
| Mineral mixture[†] | 40 | 40 |
| Vitamin mixture[‡] | 20 | 20 |
| Cellulose | 35 | 35 |
| Cholesterol | 5 | - |
| Oxidized Cholesterol | - | 5 |
| DL methionine | 2 | 2 |

[*]100 g/kg coconut oil or 100 g/kg salmon oil
[†]Mineral mixture supplied the following (per kg diet): 7.56 g calcium carbonate; 8.67 g dicalcium phosphate; 6.87 g potassium chloride; 3.77 g sodium bicarbonate; 1.01 g magnesium oxide; 0.116 g

ferrous sulfate hydrate; 0.038 g zinc oxide; 0.016 g manganese oxide; 0.024 g copper sulfate pentahydrate; 0.0032 g calcium iodate; 0.0033 g sodium selenite pentahydrate; saccharose to 40 g. ‡Vitamin mixture supplied the following (per kg diet): 1.34 mg all-*trans*-retinol; 25 µg cholecalciferol; 7.5 mg menadion sodium bisulfite; 5 mg thiamine hydrochloride; 6 mg riboflavine; 6 mg pyridoxine hydrochloride; 15 mg calcium pantothenate; 30 mg nicotinic acid; 1,000 mg choline chloride; 2 mg folic acid; 0.2 mg biotin; 0.025 mg vitamin B12; saccharose to 20 g.

To equalize the vitamin E concentrations of the diets irrespective of the dietary fats used, the native tocopherol concentrations of the two fats were analysed. Based on these native concentrations, diets were supplemented individually with all-*rac*-α-tocopheryl acetate (Merck Eurolab, Darmstadt, Germany), allowing for a biopotency of 67% compared to α-tocopherol. The final vitamin E concentrations of both types of diets were 40 mg α-tocopherol equivalents/kg. The fatty acid composition of coconut oil (Palmin, Hamburg, Germany) and salmon oil (Caelo, Hilden, Germany) is shown in Table 2.

**Table 2**        Fatty acid composition of the dietary fats[*]

| Fatty acid | Coconut oil (g/100 g fatty acids) | Salmon oil (g/100 g fatty acids) |
|---|---|---|
| 8:0 | 8.1 | -[†] |
| 10:0 | 6.3 | -[†] |
| 12:0 | 45.6 | -[†] |
| 14:0 | 17.0 | 6.1 |
| 16:0 | 9.7 | 14.5 |
| 16:1 | -[†] | 8.2 |
| 18:0 | 4.5 | 3.0 |
| 18:1 (n - 9) | 6.3 | 12.9 |
| 18:1 (n - 7) | -[†] | 2.7 |
| 18:2 (n - 6) | 1.9 | 2.2 |
| 20:1 | -[†] | 4.3 |
| 20:4 (n - 3) | -[†] | 1.4 |
| 20:5 (n - 3) | -[†] | 12.7 |
| 22:1 | -[†] | 4.5 |
| 22:5 (n - 3) | -[†] | 3.1 |
| 22:6 (n - 3) | -[†] | 10.0 |

[*]The table contains fatty acids with amounts greater than 1 g/100 g fatty acids only.
[†]Fatty acid exists in amounts smaller than 1 g/100 g fatty acids.

The peroxide values of the dietary fats, which were extracted from the diets with a mixture of hexane and isopropanol (3:2, as described in [16]) and measured according to official methods [17], were 3.9 and <0.1 mEq $O_2$ per kg salmon oil and coconut oil, respectively.

The diets were prepared weekly by solubilizing the all-*rac*-α-tocopheryl acetate and pure cholesterol/oxidized cholesterol preparation in the fat and mixing it with the dry components and water. The diets were then freeze dried and stored at −20°C to prevent autoxidation of lipids, e.g. polyunsaturated fatty acids and cholesterol. The water content after freeze drying was below 5 g per 100 g of diet.

To standardize the feed intake, the diets were fed daily in restricted amounts at 0800 h. The feed intake was increased from 7.0 g per day to 15.0 g per day during the experiment resulting in an average daily feed intake of 14.4 g. Water was provided ad libitum from nipple drinkers. The experimental diets were fed for 35 days.

*Sample collection*
At the end of the feeding period the rats were starved overnight, anesthetized with diethyl ether and killed by decapitation. The liver was excised immediately, frozen with liquid

nitrogen and stored at -80°C until analysis. Plasma was separated from blood by centrifugation (1,100 g, 10 min) at 4°C. For separation of VLDL the plasma density was adjusted to δ=1006 g/L by adding 0.3 mL of a solution containing 0.195 mol/L sodium chloride and 2.44 mol/L sodium bromide to 0.6 mL of plasma and centrifuged using a Mikro-Ultracentrifuge (Sorvall Products, Bad Homburg, Germany) at 900,000 g for 1.5 h. Plasma and VLDL were stored at –80°C until analysis.

*Lipid analysis*
Liver lipids were extracted with a mixture of hexane and isopropanol (3:2, v/v) [16]. Total cholesterol and triglyceride concentrations of liver, plasma, and VLDL were determined using enzymatic reagent kits obtained from Merck Eurolab (Darmstadt, Germany). For the measurement of liver total cholesterol and liver triglycerides, lipids of the extract were dissolved in Triton X-100 before enzymatic measurement as described by De Hoff et al. [18]. Hepatic cardiolipin (CL), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI), phosphatidyl choline (PC) and sphingomyelin (SM) were separated using a HPLC station (Hewlett Packard, Waldbronn, Germany) fitted with a Supelcosil column (25 cm, 4.6 mm, 5 µm; Supelco, Bellefonte, USA) and quantified using an evaporative light scattering detector (Sedex 55; SEDERE, Alfortville Cedex, France) and external standards. For analysis of fatty acids of liver lipids triglycerides, PE and PC were collected with a fraction collector (FC 203B; Abimed, Langenfeld, Germany). The fatty acid composition of experimental fats and liver TG, PE and PC was determined by gas chromatography (GC) of fatty acid methyl esters (FAME) as described previously in detail [19]. Briefly, fats were methylated with trimethylsulfonium hydroxide according to [20]. FAME were separated using a GC system (HP 5890, Hewlett-Packard GmbH, Böblingen, Germany) fitted with an automatic on-column injector, a polar capillary column (30 m FFAP, 0.53 mm i.d., Macherey and Nagel, Düren, Germany) and a flame ionization detector.

*Measurement of G6PDH activity*
The activity of glucose-6-phosphate dehydrogenase (G6PDH) in the liver was determined by the method of Deutsch [21]. One unit of G6PDH is defined as one µmol reduced β-nicotinamide adenine dinucleotide phosphate oxidized per min.

*Measurement of relative mRNA concentrations*
To quantify the gene expression of apoB, MTP and SREBP-1c the total RNA was isolated from liver by Trizol[TM] reagent (Invitrogen, Karlsruhe, Germany). The relative quantities of apoB mRNA, MTP mRNA and SREBP-1c mRNA compared to GAPDH mRNA were determined by quantitative real-time RT-PCR. First strand cDNA was prepared using the Omniscript RT Kit (200) from QIAGEN (Hilden, Germany) and Oligo dT-Primer $pd(T)_{12-18}$ from Amersham Pharmacia Biotech (Freiburg, Germany). The real-time PCR was performed with a Taq DNA Polymerase from Promega (Mannheim, Germany) and specific primers coding for GAPDH, apoB, MTP and SREBP-1c. Amplification of first strand cDNA was performed using a real-time RCR cycler (Rotorgene, Corbett Research, LTF Labortechnik, Wasserburg, Germany). The primers used for PCR were as follows: 5′-GCATGGCCTTCCGTGTTCC-3′ (sense) and 5′-GGGTGGTCCAGGGTTTCTTACTC-3′ (antisense) for rat GAPDH; 5′-GGAAAGGGGAGGGAAAAGGTT-3′ (sense) and 5′-TTAGGTAGGGGCTCACATTATTGG-3′ (antisense) for rat apoB; 5′-GGAGCCATGGATTGCACATT-3′ (sense) and 5′-AGGAAGGCTTCCAGAGAGGA-3′ (antisense) for rat SREBP-1c; mouse specific 5′-CGCGAGTCTAAAACCCGAGTG-3′ (sense) and 5′-CCCTGCCTGTAGATAGCCTTTCAT-3′ (antisense) for rat MTP.

*Statistical analysis*
Data were subjected to ANOVA using the Minitab Statistical Software (Minitab, State College, PA, USA). Classification factors were dietary fat, cholesterol and the interaction of both factors. In cases where the differences between variances and means were large, data were transformed to logarithms prior to ANOVA. For statistically significant F values, individual means of the treatment groups were compared by Fisher´s multiple range test. Means were considered significantly different for $P<0.05$.

## Results

*Concentrations of oxysterols in the diets.* The diets supplemented with 5 g oxidized cholesterol/kg contained the following oxysterols (mg/kg) which are known to be formed at the highest concentrations during heating of cholesterol: 7β-OH, 2.7; α-epoxycholesterol, 2.6; β-epoxycholesterol, 6.9; cholestanetriol, 0.1; 25-hydroxycholesterol, 9.4; 7-ketocholesterol, 1.7. Other minor, unidentified oxysterols have not been quanified. In the diets containing pure cholesterol the concentrations of all oxysterols were below the limit of detection of 0.025 mg/kg.

*Body weights of the rats.* The initial body weights of the rats were similar within the four treatment groups (Table 3). However, rats fed the salmon oil diets gained significantly more body weight during the feeding period and had significantly higher final body weights than the rats fed coconut oil. The type of cholesterol (oxidized vs. pure cholesterol) did not affect the body weight gain and the final body weights of the rats.

**Table 3**          Body weights, body weight gain and concentrations of plasma and VLDL lipids of rats fed diets with two different dietary fats (coconut oil vs. salmon oil) and with 5 g/kg of either pure or oxidized cholesterol

| Dietary Fat | Coconut oil | | Salmon oil | |
|---|---|---|---|---|
| Dietary Cholesterol | Pure Cholesterol | Oxidized Cholesterol | Pure Cholesterol | Oxidized Cholesterol |
| Parameter | ($n$ = 9) | ($n$ = 9) | ($n$ = 9) | ($n$ = 9) |
| Initial body weight (g) | 72.0 ± 8.2 | 72.2 ± 8.0 | 72.3 ± 8.3 | 71.9 ± 9.1 |
| Final body weight (g)[*] | 261 ± 13[b] | 265 ± 10[b] | 284 ± 10[a] | 283 ± 11[a] |
| Body weight gain (g/d)[*] | 5.41 ± 0.34[b] | 5.52 ± 0.21[b] | 6.05 ± 0.22[a] | 6.04 ± 0.36[a] |
| Triglycerides (mmol/L) | | | | |
|    Plasma[*†] | 0.79 ± 0.25[a] | 0.59 ± 0.12[b] | 0.54 ± 0.13[b] | 0.44 ± 0.19[b] |
|    VLDL[*†] | 0.21 ± 0.08[a] | 0.13 ± 0.04[b] | 0.12 ± 0.04[bc] | 0.07 ± 0.03[c] |
| Cholesterol (mmol/L) | | | | |
|    Plasma[*†] | 4.80 ± 0.47[a] | 4.45 ± 0.63[a] | 1.94 ± 0.25[b] | 1.68 ± 0.17[b] |
|    VLDL[*†‡] | 3.18 ± 0.38[a] | 2.40 ± 0.66[b] | 0.45 ± 0.14[c] | 0.34 ± 0.16[c] |

Results are expressed as means ± SD.
Results of analysis of variance: [*]Significant effect of factor fat ($P < 0.05$); [†]Significant effect of factor cholesterol ($P < 0.05$); [‡]Significant interaction between factors fat and cholesterol ($P < 0.05$). Mean values in a row without a common superscript letter differ ($P < 0.05$).
VLDL-very low density lipoprotein.

*Plasma and VLDL lipids.* The lipid concentrations of plasma and VLDL were significantly influenced by both dietary factors, the type of cholesterol and the type of fat (Table 3). The results of ANOVA showed that rats fed the diets containing oxidized cholesterol had significantly lower concentrations of triglycerides and cholesterol in plasma and VLDL than

rats fed diets containing pure cholesterol. Rats whose diets contained coconut oil had significantly higher concentrations of triglycerides and cholesterol in plasma and VLDL than rats whose diets contained salmon oil.

*Parameters of hepatic lipogenesis and concentrations of liver lipids.* The relative concentrations of SREBP-1c mRNA and the activities of G-6-PDH were both affected by the type of dietary fat (Table 4); rats fed the salmon oil diets had markedly lower relative concentrations of SREBP-1c mRNA and activities of G-6-PDH than rats fed the coconut oil diets. The type of cholesterol did not influence the relative concentrations of SREBP-1c mRNA and the activities of G-6-PDH. In contrast, neither the type of fat nor the type of cholesterol influenced the concentrations of total cholesterol, triglycerides and phospholipids in the liver.

**Table 4**        Parameters of hepatic lipogenesis and concentrations of liver lipids of rats fed diets with two different dietary fats (coconut oil vs. salmon oil) and with 5 g/kg of either pure or oxidized cholesterol

| Dietary Fat | Coconut oil | | Salmon oil | |
|---|---|---|---|---|
| Dietary Cholesterol | Pure Cholesterol | Oxidized Cholesterol | Pure Cholesterol | Oxidized Cholesterol |
| Parameter | ($n$ = 9) | ($n$ = 9) | ($n$ = 9) | ($n$ = 9) |
| SREBP-1c mRNA (% of GAPDH*100)[*] | $8.15 \pm 4.03^a$ | $7.26 \pm 3.70^a$ | $1.90 \pm 0.91^b$ | $1.27 \pm 0.45^b$ |
| G-6-PDH activity (U/mg protein)[*] | $6.94 \pm 2.38^a$ | $8.21 \pm 3.93^a$ | $1.07 \pm 0.24^b$ | $1.22 \pm 0.28^b$ |
| | | (µmol/g) | | |
| Total cholesterol | 103 ± 28 | 111 ± 24 | 125 ± 22 | 133 ± 25 |
| Triglycerides | 26.6 ± 5.6 | 35.6 ± 11.4 | 38.0 ± 11.5 | 41.7 ± 19.7 |
| Cardiolipin | 0.77 ± 0.15 | 0.80 ± 0.12 | 0.68 ± 0.10 | 0.69 ± 0.09 |
| PE | 11.9 ± 0.7 | 12.3 ± 0.6 | 11.7 ± 1.1 | 12.0 ± 1.2 |
| PI | 4.98 ± 0.41 | 4.72 ± 0.46 | 4.62 ± 0.50 | 4.34 ± 0.77 |
| PC | 17.7 ± 1.5 | 17.6 ± 1.2 | 18.5 ± 1.4 | 17.5 ± 2.0 |
| Sphingomyelin | 3.07 ± 1.05 | 2.86 ± 0.44 | 2.33 ± 0.32 | 2.65 ± 0.65 |

Results are expressed as means ± SD.
Results of analysis of variance: [*]Significant effect of factor fat ($P < 0.05$). Mean values in a row without a common superscript letter differ ($P < 0.05$).
SREBP-1c-sterol regulatory element binding protein-1c. G-6-PDH-glucose-6-phosphat dehydrogenase. PE-phosphatidylethanolamine. PI-phosphatidylinositol. PC-phosphatidylcholine.

*Fatty acid composition of liver lipids.* The fatty acid compositions of liver TG, PE and PC were strongly influenced by the type of dietary fat (Tables 5-7). Rats fed salmon oil diets had significantly higher proportions of long chain n-3 PUFA and total PUFA, but significantly lower proportions of total MUFA in TG, PE and PC than the rats fed coconut oil diets. Proportions of total SFA were slightly elevated in PE and PC, but strongly reduced in TG of the rats fed salmon oil compared to the rats fed coconut oil. Oxidized cholesterol showed only slight effects on the fatty acid composition of hepatic TG, PE and PC as compared to pure cholesterol. The only notable effect of dietary oxidized cholesterol was exhibited on the proportion of 20:4 n-6 in hepatic TG, PE and PC. Rats fed oxidized cholesterol had significantly higher proportions of 20:4 n-6 in TG, PE and PC than rats fed pure cholesterol.

**Table 5**     Fatty acid composition of liver triglycerides of rats fed diets with two different dietary fats (coconut oil vs. salmon oil) and with 5 g/kg of either pure or oxidized cholesterol

| Dietary Fat | Coconut oil | | Salmon oil | |
|---|---|---|---|---|
| Dietary Cholesterol | Pure Cholesterol | Oxidized Cholesterol | Pure Cholesterol | Oxidized Cholesterol |
| Parameter | (n = 9) | (n = 9) | (n = 9) | (n = 9) |
| Total SFA[*] | $33.7 \pm 2.0^a$ | $33.3 \pm 1.1^a$ | $16.0 \pm 1.3^b$ | $16.6 \pm 1.3^b$ |
| 16:0[*] | $28.1 \pm 2.0^a$ | $27.5 \pm 1.4^a$ | $13.1 \pm 1.4^b$ | $13.8 \pm 1.3^b$ |
| 18:0[*] | $1.76 \pm 0.28^a$ | $1.71 \pm 0.17^{ab}$ | $1.50 \pm 0.15^b$ | $1.48 \pm 0.22^b$ |
| Total MUFA[*] | $46.9 \pm 2.9^a$ | $48.9 \pm 1.7^a$ | $34.7 \pm 1.2^b$ | $34.1 \pm 1.8^b$ |
| 16:1[*] | $12.0 \pm 1.3^a$ | $12.9 \pm 0.6^a$ | $7.84 \pm 1.15^b$ | $7.53 \pm 0.99^b$ |
| 18:1[*†‡] | $34.1 \pm 1.8^b$ | $37.0 \pm 2.5^a$ | $25.8 \pm 1.4^c$ | $25.7 \pm 1.2^c$ |
| Total PUFA[*] | $2.94 \pm 0.50^b$ | $2.98 \pm 0.36^b$ | $35.3 \pm 1.3^a$ | $34.2 \pm 1.3^a$ |
| 18:2n-6[*†] | $1.35 \pm 0.25^c$ | $1.57 \pm 0.16^c$ | $4.15 \pm 0.43^b$ | $4.63 \pm 0.34^a$ |
| 20:2n-6[*] | $0.36 \pm 0.08^a$ | $0.34 \pm 0.05^a$ | $0.13 \pm 0.10^b$ | $0.14 \pm 0.11^b$ |
| 20:3n-6[*] | $0.09 \pm 0.03^b$ | $0.09 \pm 0.03^b$ | $0.22 \pm 0.03^a$ | $0.24 \pm 0.04^a$ |
| 20:4n-6[*†] | $0.41 \pm 0.06^b$ | $0.47 \pm 0.09^b$ | $0.62 \pm 0.07^a$ | $0.69 \pm 0.12^a$ |
| 20:5n-3[*] | $0.14 \pm 0.07^b$ | $0.10 \pm 0.05^b$ | $6.61 \pm 0.86^a$ | $6.87 \pm 1.01^a$ |
| 22:5n-3[*] | $0.21 \pm 0.12^b$ | $0.13 \pm 0.05^b$ | $4.81 \pm 0.69^a$ | $4.50 \pm 0.60^a$ |
| 22:6n-3[*] | $0.26 \pm 0.14^b$ | $0.12 \pm 0.03^b$ | $18.2 \pm 1.0^a$ | $17.4 \pm 1.4^a$ |

Results are expressed as means ± SD.
Results of analysis of variance: [*]Significant effect of factor fat ($P < 0.05$); [†]Significant effect of factor cholesterol ($P < 0.05$); [‡]Significant interaction between factors fat and cholesterol ($P < 0.05$). Mean values in a row without a common superscript letter differ ($P < 0.05$).
SFA-saturated fatty acids. MUFA-unsaturated fatty acids. PUFA-polyunsaturated fatty acids.

**Table 6**     Fatty acid composition of liver phosphatidylethanolamine of rats fed diets with two different dietary fats (coconut oil vs. salmon oil) and with 5 g/kg of either pure or oxidized cholesterol

| Dietary Fat | Coconut oil | | Salmon oil | |
|---|---|---|---|---|
| Dietary Cholesterol | Pure Cholesterol | Oxidized Cholesterol | Pure Cholesterol | Oxidized Cholesterol |
| Parameter | (n = 9) | (n = 9) | (n = 9) | (n = 9) |
| Total SFA[*†] | $31.8 \pm 1.0^b$ | $32.4 \pm 0.7^b$ | $32.2 \pm 1.6^b$ | $33.7 \pm 1.0^a$ |
| 16:0 | $14.4 \pm 0.8$ | $14.5 \pm 0.9$ | $15.5 \pm 1.05$ | $14.9 \pm 0.9$ |
| 18:0 | $16.9 \pm 1.3$ | $17.5 \pm 1.2$ | $16.9 \pm 1.5$ | $18.4 \pm 1.0$ |
| Total MUFA[*†] | $13.3 \pm 0.8^a$ | $12.8 \pm 1.2^a$ | $11.5 \pm 0.7^b$ | $10.4 \pm 1.2^c$ |
| 16:1[*†] | $1.95 \pm 0.25^a$ | $1.73 \pm 0.13^b$ | $1.38 \pm 0.17^c$ | $1.15 \pm 0.20^d$ |
| 18:1[*] | $5.46 \pm 0.57^a$ | $5.38 \pm 0.64^a$ | $4.94 \pm 0.24^{ab}$ | $4.62 \pm 0.74^b$ |
| Total PUFA[*†] | $39.8 \pm 0.7^c$ | $40.6 \pm 0.9^{bc}$ | $41.5 \pm 1.6^b$ | $42.7 \pm 0.7^a$ |
| 18:2n-6[*] | $5.15 \pm 0.53^a$ | $5.30 \pm 0.43^a$ | $2.29 \pm 0.27^b$ | $2.53 \pm 0.21^b$ |
| 20:2n-6[*] | $2.91 \pm 0.55^a$ | $3.11 \pm 0.45^a$ | $0.25 \pm 0.22^b$ | $0.22 \pm 0.22^b$ |
| 20:3n-6[*] | $1.21 \pm 0.13^a$ | $1.24 \pm 0.13^a$ | $0.47 \pm 0.07^b$ | $0.51 \pm 0.07^b$ |
| 20:4n-6[*†] | $20.6 \pm 1.4^b$ | $21.9 \pm 0.6^a$ | $7.18 \pm 0.73^d$ | $8.09 \pm 0.79^c$ |
| 20:5n-3[*] | $0.60 \pm 0.09^b$ | $0.66 \pm 0.11^b$ | $11.2 \pm 0.60^a$ | $11.5 \pm 0.93^a$ |
| 22:5n-3[*] | $0.34 \pm 0.15^b$ | $0.33 \pm 0.07^b$ | $3.01 \pm 0.33^a$ | $2.86 \pm 0.48^a$ |
| 22:6n-3[*] | $7.59 \pm 0.78^b$ | $7.16 \pm 0.47^b$ | $16.4 \pm 1.50^a$ | $15.8 \pm 1.04^a$ |

Results are expressed as means ± SD.
Results of analysis of variance: [*]Significant effect of factor fat ($P < 0.05$); [†]Significant effect of factor cholesterol ($P < 0.05$). Mean values in a row without a common superscript letter differ ($P < 0.05$).
SFA-saturated fatty acids. MUFA-unsaturated fatty acids. PUFA-polyunsaturated fatty acids.
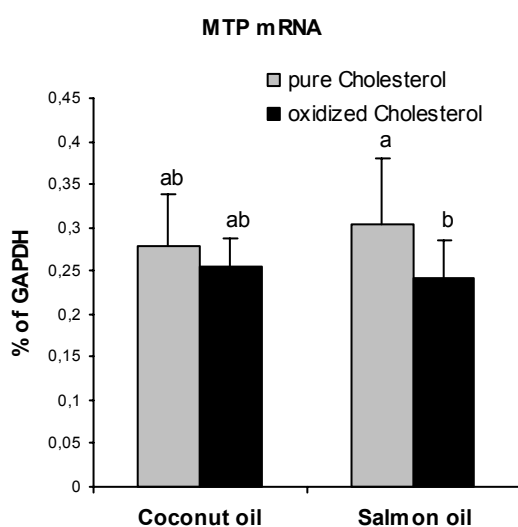
**Table 7**        Fatty acid composition of liver phosphatidylcholine of rats fed diets with two different dietary fats (coconut oil vs. salmon oil) and with 5 g/kg of either pure or oxidized cholesterol

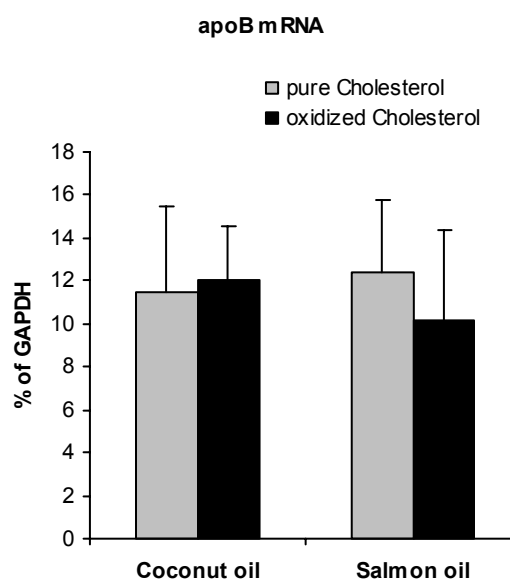| Dietary Fat | Coconut oil | | Salmon oil | |
|---|---|---|---|---|
| Dietary Cholesterol | Pure Cholesterol | Oxidized Cholesterol | Pure Cholesterol | Oxidized Cholesterol |
| Parameter | ($n$ = 9) | ($n$ = 9) | ($n$ = 9) | ($n$ = 9) |
| Total SFA[*] | $36.8\pm0.9^b$ | $37.2\pm1.1^b$ | $38.0\pm1.2^{ab}$ | $38.3\pm0.8^a$ |
| 16:0[*†‡] | $16.7\pm0.7^c$ | $17.1\pm0.9^c$ | $21.7\pm1.1^a$ | $20.3\pm0.9^b$ |
| 18:0[*†‡] | $19.5\pm1.2^a$ | $19.4\pm1.2^a$ | $15.6\pm1.2^c$ | $17.4\pm1.0^b$ |
| Total MUFA[*†] | $20.0\pm1.2^a$ | $19.3\pm1.2^a$ | $17.4\pm0.8^b$ | $16.0\pm1.2^c$ |
| 16:1[*†] | $3.77\pm0.46^a$ | $3.44\pm0.39^{ab}$ | $3.16\pm0.37^b$ | $2.66\pm0.35^c$ |
| 18:1[*] | $10.3\pm0.5^a$ | $10.5\pm0.4^a$ | $9.43\pm0.52^b$ | $9.21\pm0.93^b$ |
| Total PUFA[*] | $33.1\pm0.7^b$ | $33.8\pm1.0^b$ | $37.2\pm0.8^a$ | $37.5\pm0.8^a$ |
| 18:2n-6[*] | $9.43\pm0.65^a$ | $9.53\pm0.71^a$ | $3.98\pm0.41^b$ | $4.32\pm0.29^b$ |
| 20:2n-6[*] | $6.20\pm0.88^a$ | $6.29\pm0.66^a$ | $0.26\pm0.12^b$ | $0.27\pm0.12^b$ |
| 20:3n-6[*] | $2.56\pm0.19^a$ | $2.50\pm0.22^a$ | $1.21\pm0.12^b$ | $1.28\pm0.11^b$ |
| 20:4n-6[*†] | $11.5\pm0.5^{ab}$ | $12.0\pm1.1^a$ | $9.83\pm0.50^c$ | $11.1\pm0.3^b$ |
| 20:5n-3[*†‡] | $0.31\pm0.10^c$ | $0.34\pm0.05^c$ | $11.3\pm0.6^a$ | $10.3\pm0.7^b$ |
| 22:5n-3[*] | $0.11\pm0.02^b$ | $0.13\pm0.02^b$ | $2.06\pm0.19^a$ | $1.90\pm0.26^a$ |
| 22:6n-3[*] | $2.44\pm0.31^b$ | $2.34\pm0.09^b$ | $7.80\pm0.49^a$ | $7.61\pm0.51^a$ |

Results are expressed as means ± SD.
Results of analysis of variance: [*]Significant effect of factor fat ($P < 0.05$); [†]Significant effect of factor cholesterol ($P < 0.05$); [‡]Significant interaction between factors fat and cholesterol ($P < 0.05$). Mean values in a row without a common superscript letter differ ($P < 0.05$).
SFA-saturated fatty acids. MUFA-unsaturated fatty acids. PUFA-polyunsaturated fatty acids.

**MTP mRNA**



**apoB mRNA**



**Figure 1**        Relative concentration of microsomal triglyceride transfer protein (MTP) mRNA (MTP mRNA in % of GAPDH) of rats fed diets with two different dietary fats (coconut oil vs. salmon oil) and with 5 g/kg of either pure or oxidized cholesterol. Results are means ± SD of nine rats per group. Results of analysis of variance: Significant effect of factor cholesterol ($P < 0.05$). Bars marked without a common superscript letter differ ($P < 0.05$).

**Figure 2**        Relative concentration of apolipoprotein B (apoB) mRNA (apoB mRNA in % of GAPDH) of rats fed diets with two different dietary fats (coconut oil vs. salmon oil) and with 5 g/kg of either pure or oxidized cholesterol. Results are means ± SD of nine rats per group.

*Relative mRNA concentrations of apoB and MTP.* According to ANOVA, the relative concentrations of MTP mRNA were significantly influenced by the factor cholesterol (Figure

1). Rats receiving oxidized cholesterol had lower relative concentrations of MTP mRNA than rats receiving pure cholesterol. The type of fat showed no effect on the relative concentrations of MTP mRNA. The relative concentrations of apoB mRNA were not affected by both dietary factors investigated, the type of fat and type of cholesterol (Figure 2).

**Discussion**

In this study the effect of dietary oxidized cholesterol compared to pure cholesterol on lipid metabolism in rats was investigated with special regard to parameters of VLDL synthesis and secretion. To gain insight into the composition and the degree of oxidation of the oxidized cholesterol preparation we measured six of the dominating oxysterols knowing to be formed under the applied oxidizing conditions. The measured oxysterols in the oxidized cholesterol preparation are the quantitatively dominating oxysterols in Western foods [22]. Regarding the low concentrations of those oxysterols suggests that we used a moderately oxidized preparation. This is relevant with respect to human diets concerning the estimation that about 1% of the cholesterol consumed in a mixed Western diet is oxidized [22]. The experimental animals were fed a restrictive diet in order to exclude secondary effects which might result from different feed intakes. However, the rats fed salmon oil showed a higher body weight gain than the rats fed coconut oil. This effect might be attributed to the low concentrations of essential fatty acids (linoleic, α-linolenic acid) in the coconut oil, although we could not observe any signs of a deficiency of essential fatty acids e.g. raised concentrations of 20:3 (n-9). The differences in body weights between the rats are however unlikely to jeopardize the results of our study with regard to the effects of oxidized cholesterol.

It is well documented in rats and other animal species that oxysterols of an oxidized cholesterol preparation are readily absorbed from the intestine and transported to the liver, where they exert various biological effects [4, 23]. Our study showed that feeding oxidized cholesterol lowered the concentrations of plasma cholesterol and triglycerides as observed in feeding experiments from other investigators [3-5]. The experiment further revealed that dietary oxysterols reduced the concentrations of VLDL cholesterol and triglycerides possibly indicating a reduced VLDL synthesis and/or secretion. It is known that a decreased expression of apoB and MTP reduces VLDL assembly and secretion [7]. Thus, we measured the expression of these two genes and found that the expression of MTP was significantly reduced in the rats fed oxidized cholesterol, whereas the expression of apoB was not influenced. In patients with the recessive disorder abetalipoproteinemia the genetic loss of the expression of the MTP gene is responsible for the abnormally lowered VLDL concentrations [24]. Therefore, it appears to be possible, that the reduced transcription of the MTP gene is at least in part responsible for the lowered plasma and VLDL lipid concentrations in the rats fed oxidized cholesterol.

Dietary fish oil, as compared to coconut oil, is a source of oxidative stress due to the incorporation of highly oxidation susceptible n-3 PUFA into liver lipids [25]. According to ANOVA, the effect of oxidized cholesterol on the relative gene transcription of MTP was independent of the dietary fats used. This suggests that the effect of oxidized cholesterol on MTP gene transcription is not due to the induction of oxidative stress.

The observation that hepatic concentrations of lipids and the parameters of hepatic lipogenesis (SREBP-1c mRNA, activity of G-6-PDH) were not influenced by the dietary oxidized cholesterol varies from results from other investigators [1, 2, 5]. However, this difference might be attributed to the fact that we, in contrast to those investigators, used a moderately oxidized cholesterol preparation. Oxysterols are potent inhibitors of hepatic HMG-CoA reductase and the high amount of oxysterols in the diets from those investigators might have strongly suppressed cholesterol biosynthesis in the liver [26]. The increased proportion of 20:4 n-6 in liver TG, PE and PC in the rats fed oxidized cholesterol possibly indicates a raised desaturation of linoleic acid. This is consistent with the observation from

other studies, that dietary oxidized cholesterol increases the activity of Δ6-desaturase [1, 2, 12].

Therefore, the data of our study suggest, that dietary oxidized cholesterol possibly affects VLDL assembly and/or secretion by reducing the transcription of MTP but does not impair the synthesis of component lipids. The reduced transcription of MTP after feeding oxidized cholesterol may be in part explained by the results from *in vitro* studies, which showed that the expression of cholesterol-7α-hydroxylase (CYP7A1) also affects VLDL assembly and secretion due to a positive correlation between CYP7A1 mRNA and MTP mRNA [10]. The activity of CYP7A1 was reported to be decreased by dietary oxidized cholesterol as compared to pure cholesterol [1, 2, 5]. As the activity of CYP7A1 is highly correlated with the abundance of CYP7A1 mRNA it seems possible that a decreased transcription of CYP7A1 is responsible for the reduced concentrations of MTP mRNA in this study. In addition, results from *in vitro* study showed, that the oxysterols 25-hydroxycholesterol and 7-ketocholesterol, both of which were found in the oxidized cholesterol-supplemented diets, reversed the CYP7A1-induced changes on VLDL assembly and secretion [27]. However, this explanation is speculative and has to be confirmed by following studies.

In conclusion, the study showed that dietary oxidized cholesterol significantly reduced plasma and VLDL lipid concentrations. It was further shown that dietary oxidized cholesterol lowered the expression of MTP, which is essential for VLDL assembly and secretion. However, further studies are necessary to confirm a causal linkage between dietary oxidized cholesterol and a decreased expression of MTP and lipid lowering in plasma.

### References

[1]     Osada K, Kodama T, Yamada K, Nakamura S, Sugano M. Dietary oxidized cholesterol modulates cholesterol metabolism and linoleic acid desaturation in rats fed high-cholesterol diets. Lipids 1998;33:757-764.

[2]     Osada K, Kodama T, Noda S, Yamada K, Sugano M. Oxidized cholesterol modulates age-related change in lipid metabolism in rats. Lipids 1995;30:405-413.

[3]     Cho BHS, Egwim PO, Fahey GC. Effects of pure and auto-oxidized forms of cholesterol on plasma, liver lipids and hepatic lipogenesis in chicks. Comp Biochem Physiol 1986;83B:767-770.

[4]     Vine DF, Mamo JCL, Beilin LJ, Mori TA, Croft KD. Dietary oxysterols are incorporated in plasma triglyceride-rich lipoproteins, increase their susceptibility to oxidation and increase aortic cholesterol concentration of rabbits. J Lipid Res 1998;39:1995-2004.

[5]     Osada K, Kodama T, Cui L, Ito Y, Sugano M. Effects of dietary oxidized cholesterol on lipid metabolism in differently aged rats. Biosci Biotech Biochem 1994;58:1062-1069.

[6]     Bennett AJ, Bruce JS, Salter AM, White DA, Billett MA. Hepatic microsomal triglyceride transfer protein messenger RNA concentrations are increased by dietary cholesterol in hamsters. FEBS Lett 1996;394:247-250.

[7]     Wang S, McLeod RS, Gordon DA, Yao Z. The Microsomal triglyceride transfer protein facilitates assembly and secretion of apolipoprotein B-containing lipoproteins and decreases cotranslational degradation of apolipoprotein B in transfected COS-7 cells. J Biol Chem 1996;271:14124-14133.

[8]     Hagan DL, Kienzle B, Jamil H, Hariharan N. Transcriptional regulation of human and hamster microsomal triglyceride transfer protein genes. Cell type-specific expression and response to metabolic regulators. J Biol Chem 1994;269:28737-28744.

[9]     Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. Nature 1996;383: 728-731.

[10]    Wang S-L, Du EZ, Martin D, Davis RA. Coordinate regulation of lipogenesis, the assembly and secretion of apolipoprotein B-containing lipoproteins by sterol response element binding protein 1. J Biol Chem 1997;272:19351-19358.

[11]    Kim HJ, Takahashi M, Ezaki O. Fish oil feeding decreases mature sterol regulatory element-binding protein 1 (SREBP-1) by down-regulation of SREBP-1c mRNA in mouse liver. A possible mechanism for down-regulation of lipogenic enzyme mRNAs. J Biol Chem 1999;274:25892-25898.

[12]    Osada K, Kodama T, Minehira K, Yamada K, Sugano M. Dietary protein modifies oxidized cholesterol-induced alterations of linoleic acid and cholesterol metabolism in rats. J Nutr 1996;126:1635-1643.

[13]    Reeves PG, Nielsen FH, Fahey GC. AIN-93 Purified diets for laboratory rodents: Final report of the American Institute of Nutrition ad hoc Writing Committee on the reformulation of the AIN-76A rodent diet. J Nutr 1993;123:1939-1951.

[14]    Mori TA, Croft KD, Puddey IB, Beilin LJ. Analysis of native and oxidized low-density lipoprotein oxysterols using gas chromatography-mass spectrometry with selective ion monitoring. Redox Report 1996;2:25-34.

[15]    Brandsch C, Ringseis R, Eder K. High dietary iron concentrations enhance the formation of cholesterol oxidation products in the liver of adult rats fed salmon oil with minimal effects on antioxidant status. J Nutr 2002;132:2263-2269.

[16]    Hara A, Radin NS. Lipid extraction of tissues with a low toxicity solvent. Anal Biochem 1978;90:420-426.

[17]    Association of Official Analytical Chemists. In: Horwitz H. editor. Official Methods of Analysis. 13th ed. Airlington: VA. 1980. pp. 440-441.

[18]    De Hoff JL, Davidson JH, Kritchevsky V. An enzymatic assay for determining free and total cholesterol in tissues. Clin Chem 1978;24:433-435.

[19]    Brandsch C., Ringseis R., Eder K. High dietary iron concentrations enhance the formation of cholesterol oxidation products in the liver of adult rats fed salmon oil with minimal effects on antioxidant status. J Nutr 2002;132:2263-2269.

[20]    Butte W. Rapid method for the determination of fatty acid profiles from fats and oils using trimethylsulfonium hydroxide for transesterification. J Chromatogr 1983;261:142-145.

[21]    Deutsch J. (1985) Glucose-6-Phosphat-Dehydrogenase. In: Bergmeyer HU. Editor. Methods of enzymatic analysis. Vol. 3. Weinheim: VCH. 1985. pp. 190-197.

[22]    Van de Bovenkamp P, Kosmeijer-Schuil TG, Katan MB. Quantification of oxysterols in Dutch foods: egg products and mixed diets. Lipids 1988;23:1079-1085.

[23]    Vine DF, Croft KD, Beilin LJ, Mamo JCL. Absorption of dietary cholesterol oxidation products and incorporation into rat lymph chylomicrons. Lipids 1997;32:887-893.

[24]    Wetterau JR, Aggerbeck LP, Bouma ME, Eisenberg C, Munck A, Hermier M, Schmitz J, Gay G, Rader DJ, Gregg RE. Absence of microsomal triglyceride transfer protein in individuals with abetalipoproteinemia. Science 1992;258:999-1001.

[25]    D'Aquino M, Benedetti PC, Di Felice M, Gentili V, Tomassi G, Maiorino M, Ursini F. Effect of fish oil and coconut oil on antioxidant defence system and lipid peroxidation in rat liver. Free Radic Res Commun 1991;1:147-152.

[26]    Johnson KA, Morrow CJ, Knight GD, Scallen TJ. In vivo formation of 25-hydroxycholesterol from endogenous cholesterol after a single meal, dietary cholesterol challenge. J Lipid Res 1994;35:2241-2253.

[27]    Du EZ, Fleming JF, Wang S-L, Spitsen GM, Davis RA. Translocation-arrested apolipoprotein B evades proteasome degradation via a sterol-sensitive block in ubiquitin conjugation. J Biol Chem 1999;274:1856-1862.

## 4.      Diskussion

## 4.1      Nutritive Beeinflussung der Konzentrationen an 7β-Hydroxycholesterol und anderer „nicht-enzymatisch" gebildeter Oxysterole

### 4.1.1   Effekt einer hohen Zufuhr von Fischöl

Eine erhöhte Aufnahme an Fischöl zeigte beim Menschen einen protektiven Effekt vor kardiovaskulären Erkrankungen wie Arteriosklerose, Bluthochdruck, Schlaganfall und Herzinfarkt (NESTEL 1990, BURR et al. 1989, DE LORGERIL et al. 1999). Dies wird auf die im Fischöl in hohen Konzentrationen enthaltenen n-3-PUFA, insbesondere der Eicosapentaensäure zurückgeführt (HARRIS 1989, MEHTA et al. 1987). Allerdings führt eine erhöhte Aufnahme an Fischöl ohne ausreichenden antioxidativen Schutz auch zur Induzierung von oxidativem Stress (IBRAHIM et al. 1997, KUBOW et al. 1996), was auf die Inkorporierung dieser oxidationsempfindlichen n-3-PUFA in die Biomembranen im Organismus zurückzuführen ist (KIRCHGESSNER et al. 1994, MALIS et al. 1990, NALBONE et al. 1989, SAITO und NAKATSUGAWA 1994, SEN et al. 1997, STANGL et al. 1994, ZSIGMOND et al. 1990). Dadurch steigen die Oxidationssensitivität der Membranen und die endogene Lipidperoxidation an und es entsteht ein postabsorptiver Vitamin E-Verbrauch (CHO und CHOI 1994, D`AQUINO et al. 1991, HU et al. 1989, IBRAHIM et al. 1997, KUBOW et al. 1996, LEIBOWITZ et al. 1990, MEYDANI et al. 1987, MOURI et al. 1984, NALBONE et al. 1989, SAITO und NAKATSUGAWA 1994). Wie in der Studie 1 der vorliegenden Arbeit erstmalig beobachtet, führt die Verfütterung von Fischöl im Vergleich zu Schweineschmalz oder Kokosfett aber auch zu einer signifikanten Erhöhung „nicht-enzymatisch" gebildeter Oxysterole in der Leber von Ratten (BRANDSCH et al. 2002). In den Studien 2 und 3 konnte darüber hinaus gezeigt werden, dass Fischöl die Konzentrationen an 7β-OH-Chol, das als Marker für die „nicht-enzymatische" Oxysterolbildung diente, in Plasma, LDL und Erythrozyten von Ratten erhöht (RINGSEIS und EDER 2002 und 2003), was aufgrund der pathophysiologischen Bedeutung dieser Oxysterole möglicherweise als kritisch anzusehen ist.

Die signifikant verringerten α-Tocopherolkonzentrationen in den untersuchten Geweben zeigten, dass Lachsöl in allen drei Studien zu einer Verschlechterung des Vitamin E-Status führte. Dies war allerdings unabhängig vom unterschiedlichen Vitamin E-Gehalt der Diätfette, da dieser Unterschied durch Zugabe von all-*rac*-α-Tocopherylacetat zu den Diäten ausgeglichen wurde. Der Anstieg der Oxysterolkonzentrationen in den untersuchten Geweben

kann somit auf das Lachsöl, welches postabsorptiv den Vitamin E-Verbrauch erhöhte, zurückgeführt werden. Daneben bewirkte dieser prooxidative Effekt das Lachsöls einen starken Anstieg der Konzentrationen an TBARS und Glutathion (GSH) (gesamt GSH und oxidiertes GSH) in der Leber (BRANDSCH et al. 2002). Erhöhte TBARS-Konzentrationen treten bei Verabreichung von Lachsöl als Konsequenz einer erhöhten Lipidperoxidation auf (CHO und CHOI 1994, CHO et al. 1995, JAVOUHEY-DONZEL et al. 1993, VENKATRAMAN et al. 1998), während erhöhte GSH-Konzentrationen als Folge der Adaptation an den durch das Fischöl induzierten oxidativen Stress interpretiert werden (IBRAHIM et al. 1997). Insgesamt deuten diese Beobachtungen daraufhin, dass die Verabreichung von Lachsöl in den Studien 1-3 zu einer starken Induzierung von oxidativem Stress führte. Als geeignete Parameter zur Beschreibung des *in vivo*-Lipidperoxidationsgeschehens und als Marker für oxidativen Stress haben sich insbesondere 7β-OH-Chol, aber auch andere „nicht-enzymatisch" gebildete Oxysterole erwiesen (ADACHI et al. 2001, HODIS et al. 2000, ISHII et al. 2002, IULIANO et al. 2001, PORKKALA-SARATAHO et al. 2000, SALONEN et al. 1997, SKRZEP-POLOCZEK et al. 2001, ZIEDEN et al. 1999). Es lässt sich deshalb vermuten, dass die Aufnahme von Lachsöl über die Induzierung von oxidativem Stress die „nicht-enzymatische" Bildung von Oxysterolen erhöht hat.

Diese Vermutung wird auch durch *in vitro*-Untersuchungen an Modellmembranen unterstützt, die Hinweise darauf liefern konnten, dass die nicht-enzymatische Oxidation des Cholesterols in Membranen durch Peroxyl- und Alkoxylradikale erfolgt, die als Folge der Peroxidation von PUFAs auftreten (SEVANIAN und MCLEOD 1987, TERAO et al. 1985, MUTO et al. 1983). Unter anderem wurde gezeigt, dass Linolsäureradikale zur Oxygenierung des Cholesterols am C-Atom 7 führen (LUND et al. 1992, CARPENTER et al. 1990). Auf die Bedeutung der primären Oxidation von PUFAs für die Cholesteroloxidation deuten auch Beobachtungen hin, dass in einem Lipoxygenasesystem Cholesterol nur in Gegenwart von Ethyllinoleat oxidiert wird (TENG und SMITH 1973) und dass bei der *in vitro*-LDL-Oxidation der Konzentrationsabnahme an PUFAs eine Konzentrationszunahme an 7β-OH-Chol nachfolgt (CARPENTER et al. 1994). Die Bildung von Oxysterolen im Zuge der Lipidperoxidation erfolgt wahrscheinlich dadurch, dass eine Wasserstoffabstraktion an der oxidationsempfindlichen alpha-Methylengruppe Gruppe der PUFA und nachfolgende Anlagerung von Sauerstoff zur Bildung eines Fettsäureperoxylradikals führt (FRANKEL 1991, GUTTERIDGE 1995). Dieses Fettsäureperoxylradikal kann nun ein Wasserstoffatom

an der empfindlichen allylischen C7-Position des Cholesterols abstrahieren, woraus primär die thermodynamisch instabilen, epimeren 7-Hydroperoxycholesterole (7-OOH-Chol) entstehen, welche über verschiedene Mechanismen wie Radikal-Kombinierung und/oder – Disproportionierung zu den stabileren Endprodukten 7α- und 7β-OH-Chol und 7-Keto-Chol weiter reagieren (GARDNER 1987). Darauf deutet unter anderem die 7-OOH-Chol-Bildung in der Frühphase und die 7-OH-Chol-Bildung in der Spätphase der LDL-Oxidation hin (BROWN et al. 1997).

Auch die C-Atome 5 und 6 des Cholesterols sind Ziele eines radikalischen Angriffs. Dies führt zur Bildung der epimeren Cholesterolepoxide, die weiter zu Cholestanetriol reagieren können (BROWN et al. 1997, CARPENTER et al. 1994, LINSEISEN und WOLFRAM 1998b). Hinweise auf eine ROS-induzierte Bildung der Cholesterolepoxide und des Cholestanetriols liefern Beobachtungen aus tierexperimentellen Studien, dass deren Gehalte in Plasma und Aortengewebe durch Verabreichung von Antioxidantien vermindert wurden (BJÖRKHEM et al. 1991, HODIS et al. 1992), sowie der Befund, dass diese Oxysterole während der *in vitro*-Oxidation von LDL gebildet werden (BREUER et al. 1996, BROWN et al. 1996, CHANG et al. 1997, DZELETOVIC et al. 1995, PATEL et al. 1996). ROS-Einwirkung an der Cholesterinseitenkette kann zur Bildung von 25-Hydroxycholesterol führen (SMITH 1996). Darauf deuten auch die infolge Lachsölfütterung erhöhten Konzentrationen in der Leber in Studie 1 hin (BRANDSCH et al. 2002). Da dieses Oxysterol aber auch auf enzymatischem Wege gebildet wird (JOHNSON et al. 1994), eignet es sich nur bedingt als Parameter der *in vivo* Lipidperoxidation.

Ein bedeutender Anteil des Cholesterols liegt im Organismus mit PUFA verestert vor. Es zeigte sich, dass verestertes Cholesterol besonders oxidationsanfällig ist, da aufgrund der Nähe der α-Methylengruppe der PUFA zu den C-Atomen 5, 6 und 7 des Cholesterols an diesen Positionen eine oxidative Modifizierung begünstigt wird (LINSEISEN und WOLFRAM 1998b). Anzeichen hierfür sind die frühe Bildung von Cholesterollinoleathydroperoxiden bei der *in vitro*-Oxidation von LDL und ein hoher Anteil an verestertem 7-OH-Chol im oxLDL (BROWN et al. 1996). In diesem Zusammenhang erscheint es bedeutsam, dass die hepatischen Cholesterolkonzentrationen durch Verabreichung von Fischöl in Studie 1 und 3 in Übereinstimmung mit anderen Untersuchungen (CHAUTAN et al. 1990, RUIZ-GUTIERREZ et al. 1999, VENTURA et al. 1989, YAQOOB et al. 1995) angestiegen sind, was auf eine infolge Fischölfütterung

gesteigerte Aktivität der ACAT zurückgeführt wird (CHAUTAN et al. 1990, FIELD et al. 1987). Es konnte mehrfach nachgewiesen werden, dass die Cholesterolkonzentrationen in der Leber positiv mit den Oxysterolkonzentrationen korrelieren (HODIS et al. 1991, OSADA et al. 1994b und 1995). Dies zeigte sich auch in dem Anstieg an 7β-OH-Chol und Cholesterol in der Leber bei Zulage von reinem Cholesterin im Vergleich zu den entsprechenden Gruppen ohne Cholesterinzulage (Exp. 2 vs. Exp. 1) in Studie 2 der vorliegenden Arbeit. Allerdings war dieser Effekt unabhängig von der Fettart zu beobachten, so dass anzunehmen ist, dass die durch Fischöl im Vergleich zu Kokosfett oder Schweineschmalz erhöhten Oxysterolkonzentrationen in der Leber in erster Linie auf den Fischöl-induzierten oxidativen Stress und nicht auf die Fischöl-bedingte Erhöhung der Cholesterolkonzentrationen zurückzuführen sein dürften. Diese Annahme wird auch dadurch unterstützt, dass die 7β-OH-Chol-Konzentrationen in Plasma und LDL, trotz erhöhter Cholesterolkonzentrationen, bei den Kokosfett-supplementierten Ratten niedriger waren.

Wie in einer Vielzahl an Studien mit Ratten bzw. Mäusen nachgewiesen werden konnte ist der Effekt des Fischöls auf die Peroxidation der PUFA abhängig von der Vitamin E-Zufuhr (CHO und CHOI 1994, CHO et al. 1995, JAVOUHEY-DONZEL et al. 1993, MEYDANI et al. 1987). Die Studie 3 ergab, dass dies auch auf die 7β-OH-Chol-Konzentrationen zutrifft. So bewirkte eine kontinuierliche Steigerung der Vitamin E-Konzentration von 10 über 20 auf 40 mg α-Tocopherol-Äquivalente (α-TÄ)/kg Diät in Plasma, LDL und Erythrozyten bei den Lachsölgefütterten Tieren eine Reduzierung der 7β-OH-Chol-Konzentration auf ein ähnliches Niveau wie bei den Kokosfettgefütterten Tieren. Allerdings war dies in der Leber nicht der Fall. Selbst bei einer hohen Vitamin E-Versorgung von 240 (Studie 2 und 3) bzw. 400 (Studie 1) mg α-TÄ/kg Diät waren die Konzentrationen an 7β-OH-Chol und anderen „nicht-enzymatisch" gebildeten Oxysterolen höher als bei Fütterung von Kokosfett oder Schweineschmalz. Möglicherweise liegt dies daran, dass die Leber das primäre Zielorgan für oxidativen Stress ist (TAKAHASHI et al. 2002), da die im Fischöl enthaltenen oxidationsanfälligen n-3-PUFA nach deren Absorption im Darm als erstes zur Leber transportiert und in die Leberlipide inkorporiert werden (NILSSON et al. 1992). Außerdem induziert Fischöl in der Leber, nicht jedoch in den Erythrozyten, die peroxisomale β-Oxidation (ATALAY et al. 2000, CHEN et al. 1993, SANDERS et al. 1985, TAKASHI et al. 2000, YEO und HOLUB 1990), bei der im Gegensatz zur mitchondrialen β-Oxidation Wasserstoffperoxid ($H_2O_2$) als Nebenprodukt entsteht (TOLBERT 1981). Vermittelt wird dies über die Aktivierung des Peroxisomen-Proliferator-Aktivierenden-Rezeptors α (PPARα)

durch n-3 PUFAs, was zu einer erhöhten Expression der $H_2O_2$-generierenden peroxisomalen Acyl-CoA-Oxidase führt (CHAO et al. 2001). Dieses membrangängige $H_2O_2$ kann über die Eisen-katalysierte Bildung von Hydroxylradikalen via Haber-Weiss-Reaktion zur Entstehung weiterer ROS beitragen (VAN DER ZEE et al. 1993), so dass möglicherweise dadurch der prooxidative Effekt des Fischöls in der Leber verstärkt wird. Ein Indiz für die PPARα-Aktivierung bei den Ratten in Studie 2 der vorliegenden Arbeit infolge der Fütterung von Fischöl ist die signifikante Transkriptionssteigerung PPARα-aktivierter Gene wie der *medium chain acyl-CoA dehydrogenase,* der *3-ketoacyl-CoA thiolase A* und *B* und der Cytochrom P450 Enzyme *CYP4A1* und *CYP4A3* im Vergleich zur Fütterung von Kokosfett, was mit Hilfe der Anwendung eines cDNA Expression Macroarrays in eigenen Studien gezeigt werden konnte (unveröffentlichte Daten). Ein weiterer Hinweis für die PPARα-Aktivierung durch Fischöl ist die beobachtete Lebervergrößerung (Hepatomegalie) in Studie 2 und 3 der vorliegenden Arbeit (Tabelle 1).

**Tab. 1:**         Relative Lebermassen bei Fütterung von Fischöl im Vergleich zu Kokosfett

| Vitamin E-Konzentration (mg α-TÄ/kg Diät) | Kokosfett | Lachsöl | P-Wert |
|---|---|---|---|
| *Studie 2 (Exp. 1)* | mg Leber/g Körpermasse | | |
| 40 | 33,8 ± 1,56 | 37,0 ± 2,79 | <0,01 |
| 240 | 33,3 ± 2,62 | 35,2 ± 2,90 | NS |
| *Studie 3* | mg Leber/g Körpermasse | | |
| 10 | 29,8 ± 1,62 | 36,3 ± 4,84 | <0,001 |
| 20 | 31,2 ± 3,35 | 32,6 ± 2,75 | NS |
| 40 | 29,9 ± 3,01 | 32,0 ± 1,40 | NS |
| 240 | 30,2 ± 2,37 | 31,5 ± 2,33 | NS |

Dargestellt sind die Mittelwerte ± Standardabweichungen für 9 Tiere pro Gruppe.
Abkürzungen: α-TÄ, α-Tocopherol-Äquivalente; NS, nicht signifikant.

Dies wurde auch von anderen Autoren beschrieben (KUBOW et al. 1996, OTTO et al. 1991 und 1992, YAQOOB et al. 1995) und ist auf eine zelluläre Hypertrophie zurückzuführen (OTTO et al. 1991), welche vermutlich in unmittelbarem Zusammenhang mit der Fischöl-induzierten Peroxisomen-Proliferation steht. Neben n-3-PUFA führen auch andere Peroxisomen-Proliferatoren wie Clofibrate oder Ciprofibrate zu einer Zunahme der Größe und Anzahl an Peroxisomen (TAKAGI et al. 1992, RAO und REDDY 1987) und zu einer

Vergrößerung der Leber (TAKAGI et al. 1992). Da diese Fischöl-induzierte Hepatomegalie durch Vitamin E-Supplementierung vermindert werden konnte, wie auch in Studie 2 und 3 beobachtet (Tabelle 1), wird angenommen, dass diese Hepatomegalie in Verbindung mit starkem hepatischen oxidativem Stress steht (BERGSETH et al. 1986, KUBOW et al. 1996).

Die Oxysterolmessung in den Erythrozyten diente zur Untersuchung der *in vivo*-Cholesteroloxidation in einer Membranfraktion, da sich die Erythrozyten-Lipide fast ausschließlich aus Membranlipiden zusammensetzen. An den signifikant erhöhten 7β-OH-Chol-Konzentrationen im Erythrozytenlipidextrakt in Studie 2 und 3 zeigte sich, dass Fischöl auch die nicht-enzymatische Oxysterolbildung in den Erythrozyten induziert. Auch dies ist vermutlich Folge der Fischöl-bedingten Inkorporierung oxidationsempfindlicher n-3 PUFAs wie sie in Studie 2 (Exp. 1) der vorliegenden Arbeit (Tabelle 2) und auch in anderen Studien beobachtet wurde (EDER et al. 2002, HAGVE et al. 1993, PALOZZA et al. 1996), zumal Erythrozyten aufgrund der hohen Hämoglobinkonzentration und des hohen Sauerstoffgehalts sehr empfindlich auf die Induzierung von oxidativem Stress reagieren (EDER et al. 2002). Unterstützt wird dieser Befund durch die Beobachtung, dass die Erythrozyten von Patienten mit Sichelzell-Anämie, bei der eine übermäßige Bildung von Superoxid-, Peroxid- und Hydroxylradikalen zu oxidativem Stress führt (HEBBEL et al. 1982), gegenüber normalen Erythrozyten erhöhte Konzentrationen an nicht-enzymatisch gebildeten Oxysterolen aufweisen (KUCUK et al. 1992, SZOSTEK et al. 1991, TENG und SMITH 1995).

**Tab. 2:**     Fettsäurezusammensetzung des Erythrozytenlipidextrakts bei Fütterung von Kokosfett im Vergleich zu Lachsöl[*]

|  | Kokosfett | Lachsöl |
|---|---|---|
|  | g/100 g Fettsäuren | |
| SFA | 39,7 ± 0,94 | 41,0 ± 1,44 |
| MUFA | 14,8 ± 0,64 | 15,3 ± 0,86 |
| PUFA | 39,1 ± 1,93 | 37,5 ± 1,36 |
|  n-6 PUFA | 35,9 ± 1,84 | 14,2 ± 0,56 |
|  n-3 PUFA | 3,19 ± 0,29 | 23,1 ± 1,12 |
| C20:5 n-3 | 0,46 ± 0,12 | 12,3 ± 0,74 |
| C22:6 n-3 | 1,65 ± 0,33 | 5,58 ± 0,52 |

[*]Aus Gründen der Übersichtlichkeit sind nur die Gruppen mit 40 mg α-Tocopherol-Äquivalenten/ kg Diät aus Studie 2 (Exp. 1) dargestellt.
Abkürzungen: SFA, gesättigte Fettsäuren (saturated fatty acids); MUFA, einfach ungesättigte Fettsäuren (monounsaturated fatty acids); PUFA, mehrfach ungesättigte Fettsäuren (polyunsaturated fatty acids).

Die Ergebnisse der durchgeführten Studien 1-3 lassen insgesamt darauf schliessen, dass Fischöl die Bildung von 7β-OH-Chol und anderer „nicht-enzymatischer" Oxysterole vermutlich als Folge einer gesteigerten Lipidperoxidation in Geweben und Lipoproteinen erhöhen. Wie in Studie 3 gezeigt, führt Fischöl insbesondere in Kombination mit einer geringen Vitamin E-Aufnahme zu einem starken Konzentrationsanstieg an 7β-OH-Chol in Geweben und Lipoproteinen, während eine ausreichende Vitamin E-Versorgung zu einer Senkung der 7β-OH-Chol-Konzentrationen auf dasselbe Level wie bei Kokosfettfütterung führt.

## 4.1.2   Effekt einer moderaten und hohen Zufuhr von Eisen

Eisenmangelanämie ist eine der in Deutschland aber auch weltweit dominierensten Nährstoff-abhängigen Mangelerscheinungen (COOK et al. 1994, DGE et al. 2000, RAMAKRISHNAN 2002). Deshalb wird häufig eine Supplementierung von Eisen empfohlen. In diesem Zusammenhang ziehen jedoch einige Autoren die Möglichkeit einer Eisenüberladung durch Nahrungssupplemente oder Fleisch in Betracht (KATO et al. 2000, MILMAN et al. 2002), obwohl es hierfür bislang keine Bestätigung gibt. Wahrscheinlicher ist, dass eine Eisenüberladung lediglich bei erblichen Erkrankungen wie Hämochromatose, Atransferrinämie und Friedreichs´sche Ataxie (SHETH und BRITTENHAM 2000) oder bei chronischem Alkoholmissbrauch auftritt (CHAPMAN et al. 1982, DUANE et al. 1992, FORD et al. 1995).

Eisen liegt im Organismus normalerweise in gebundener Form als Bestandteil von Hämoglobin (60%), Ferritin bzw. Hämosiderin (25%) sowie Myoglobin und eisenhaltigen Enzymen (15%) vor. Im Plasma wird Eisen an Transferrin gebunden transportiert, wobei nur etwa ein Drittel des Plasmatransferrins mit Eisen gesättigt ist (Eisenbindungskapazität), während der Rest als Transportreserve (latente Eisenbindungskapazität) zur Verfügung steht. Wird die totale Eisenbindungskapazität überschritten, kommt es zur Eisenüberladung, die durch eine massive Anreicherung von katalytisch aktiven Eisenionen in Leber und anderen Organen gekennzeichnet ist (BRITTON et al. 1987). Eisenionen katalysieren die Bildung hochreaktiver Hydroxylradikale via Fenton-Reaktion und beschleunigen über den Abbau von Lipidhydroperoxiden zu Alkoxyl-, Peroxyl- und anderen Radikalen die Lipidperoxidation (CROSS et al. 1987, DAVIES und SLATER 1987, DONOVAN und MENZEL 1978,

HALLIWELL und GUTTERIDGE 1986 und 1990). Dies ist auch der Grund dafür, dass eine tierexperimentell erzeugte Eisenüberladung zu massiver Lipidperoxidation führt (BACON et al. 1983a und 1983b, BRUNET et al. 1999, DABBAGH et al. 1994, DILLARD et al. 1983, DILLARD und TAPPEL 1979, HOUGLUM et al. 1990, WU et al. 1990) – insbesondere in den mitochondrialen und mikrosomalen Membranen (BACON et al. 1983a, BRITTON et al. 1987).

Auch bei genetisch bedingter Eisenüberladung bei Patienten mit Acoeruloplasminämie führt die starke Eisenanreicherung in den Geweben zu massiver Lipidperoxidation. Neben erhöhten Konzentrationen an TBARS bzw. Malondialdehyd in Serum, Cerebrospinalflüssigkeit und/oder Gehirn (MIYAJIMA et al. 1996 und 1998 und, YOSHIDA et al. 2000) wurden bei diesen Patienten auch erhöhte Konzentrationen an 7β-OH-Chol und 7-Keto-Chol in verschiedenen Gehirnabschnitten, aber auch in viszeralen Organen wie Leber, Bauchspeicheldrüse, Herz und Niere festgestellt (MIYAJIMA et al. 2001). Die Autoren vermuten, dass diese Oxysterolbildung auf eine gesteigerte, Eisen-katalysierte Lipidperoxidation zurückzuführen ist. Deshalb deutet auch die Beobachtung, dass eine erhöhte Eisenaufnahme bei Ratten einen Konzentrationsanstieg an 7β-OH-Chol, 7-Keto-Chol und Cholestanetriol in der Leber bewirkt (BRANDSCH et al. 2002), auf eine gesteigerte Lipidperoxidation als Ursache hin. Unterstützt wird dies auch durch den Befund, dass 7α-OH-Chol, 7β-OH-Chol, 7-Keto-Chol und α-Epoxycholesterol in Synaptosomen und/oder Mitochondrien bei der *in vitro*-Oxidation mittels $Fe^{2+}$-Ionen und Ascorbat gebildet werden (MUTO et al. 1983, TERAO et al. 1985, VATASSERY et al. 1997a und 1997b). Die oxidationsfördernde Wirkung freier Eisenionen spielt aber nicht nur in der Oxidation von Membranlipiden, sondern auch in der oxidativen Modifizierung von Lipoproteinen, insbesondere des LDLs, durch Endothelzellen und Macrophagen eine Rolle (HOESCHEN et al. 1997, LEAKE und RANKIN 1990). Dabei begünstigt Eisen die LDL-Oxidation nicht nur aufgrund der Bildung freier Radikale, sondern auch aufgrund der Verminderung der Antioxidantienkonzentrationen im Plasma (DABBAGH et al. 1994).

Der Anstieg der Oxysterolkonzentrationen infolge einer erhöhten Eisenzufuhr in Studie 1 der vorliegenden Arbeit war lediglich bei Verwendung von Lachsöl, nicht jedoch bei Verwendung von Schweineschmalz als Diätfett zu beobachten. Da Lachsöl als Folge der Inkorporierung der oxidationsempfindlichen n-3 PUFA zu einer Induzierung von oxidativem Stress und zu einer verstärkten Cholesteroloxidation führt (BRANDSCH et al. 2002,

RINGSEIS und EDER 2002 und 2003), lässt sich vermuten, dass der Anstieg der Eisenkonzentrationen in der Leber den prooxidativen Effekt des Fischöls forciert hat. Die Konzentrationen an TBARS wurden dagegen bei den Fischölgefütterten Tieren durch die hohe Eisenzufuhr nicht beeinflusst. Dass die Oxysterolmessung und die TBARS-Messung ein unterschiedliches Bild ergaben, liegt möglicherweise daran, dass der TBA-Test ein *in vitro*-Testverfahren ist, das demnach nur bedingt zur Charakterisierung der *in vivo* Lipidperoxidation geeignet ist. So wird mittels TBA-Test sowohl in der Probe vorhandenes als auch bei der Testdurchführung gebildetes Malondialdehyd erfasst. Darüberhinaus ist dieses Verfahren unspezifisch, da auch andere TBA-reaktive Substanzen wie Gallenpigmente, Aminosäuren und Kohlenhydrate erfasst werden (DRAPER et al. 1986, ESTERBAUER 1996, GUTTERIDGE und HALLIWELL 1990, JANERO 1990).

Bei der Eisen-induzierten Lipidperoxidation kommt möglicherweise der erhöhten $H_2O_2$-Bildung, die als Folge der PPARα-Aktivierung durch die im Fischöl enthaltenen n-3 PUFA zu beobachten war (siehe Punkt 4.1.1), eine entscheidende Bedeutung zu. Da bei erhöhten Gewebskonzentrationen an Eisen, wie in Studie 1 beobachtet, der Pool an katalytisch aktivem Eisen vergrößert ist (HALLIWELL und GUTTERIDGE 1992, TOYOKUNI 1996), kann Eisen dann die Bildung hochreaktiver Hydroxylradikale aus $H_2O_2$ und/oder die Bildung von Alkoxyl- und Peroxylradikalen aus Lipidhydroperoxiden katalysieren, was vermutlich in der Folge zur Peroxidation des Cholesterols führt. Dies ist umso erstaunlicher als die relativ geringe Erhöhung der hepatischen Eisenkonzentrationen in Studie 1 der vorliegenden Arbeit vermuten lässt, dass die Ratten nur eine relativ geringe Eisenüberladung aufwiesen, obgleich geeignete Parameter zur Beurteilung des Eisensstatus wie das Serum-Ferritin (COOK et al. 1974, KALTWASSER und WERNER 1989), anhand dessen sich der Sättigungsgrad der Eisenspeicher im Organismus erfassen lässt, nicht gemessen wurden. Im Gegensatz dazu führte die Eisenüberladung in anderen Studien zu etwa 10-fach erhöhten Eisenkonzentrationen in den Geweben (BACON et al. 1983b, DABBAGH et al. 1994, MIYAJIMA et al. 2001, NICHOLS und BACON 1989).

Insgesamt konnte gezeigt werden, dass eine hohe Eisenzufuhr über die Diät in Verbindung mit einer hohen Aufnahme an PUFA die Bildung spezifischer Oxysterole wie 7β-OH-Chol und 7-Keto-Chol, die wie bereits mehrfach erwähnt im Rahmen der Lipidperoxidation gebildet werden, in der Leber von Ratten erhöht. Dies ist möglicherweise kritisch zu bewerten, da diese Oxysterole von der Leber als Bestandteil von VLDL ausgeschleust werden

(VINE et al. 1998), aus welchen durch Einwirkung der endothelialen Lipoproteinlipase Oxysterol-haltige LDL hervorgehen. Da 7β-OH-Chol und 7-Keto-Chol neben 7β-OOH-Chol als die Komponenten mit der höchsten Toxizität im oxidativ modifizierten LDL identifiziert werden konnten (CHISOLM et al. 1994, HUGHES et al. 1994), erklärt dies möglicherweise die vielfach beschriebenen proatherogenen Wirkungen oxidierten LDLs (BERLINER und HEINECKE 1996, STEINBERG 1997a und 1997b).

### 4.1.3 Effekt einer niedrigen, ausreichenden und hohen Zufuhr von Vitamin E

Vitamin E umfasst eine Gruppe von jeweils vier Tocopherol- und Tocotrienolisomeren (α, β, γ, δ) (BRIGELIUS-FLOHÉ und TRABER 1999). Gemeinsames Kennzeichen dieser Moleküle ist, dass sie einen Chromanring mit einer freien bzw. einer veresterten Hydroxygruppe sowie eine gesättigte (Tocopherole) oder ungesättigte (Tocotrienole) Isoprenoid-Seitenkette aufweisen. Da die biologische Wirksamkeit der Tocopherol- und Tocotrienolisomere sehr unterschiedlich ist (α-:β-:γ-:δ-Tocopherol gleich 100:50:25:1; Tocotrienole nur etwa 20-30% der Tocopherolwirkung (SCHÄFER und ELMADFA 1984)) und α-Tocopherol die bedeutendste in der Natur vorkommende Verbindung mit Vitamin E-Aktivität ist, drückt man ihre gesamte Vitamin E-Wirkung in Form von α-Tocopherol-Äquivalenten (α-TÄ) oder Internationalen Einheiten (IE) aus, wobei 0,67 mg α-TÄ entweder 1 IE Vitamin E oder 1 mg all-*rac*-α-Tocopherylacetat (früher als D,L-α-Tocopherylacetat bezeichnet) entsprechen (ELMADFA 1990). In den Studien 1-3 wurden die unterschiedlichen α-Tocopherol-Konzentrationen in den Diätenfetten durch Zugabe des synthetisch hergestellten all-*rac*-α-Tocopherylacetats - dies stellt eine Mischung aus acht Vitamin E-aktiven Isomeren dar - zu den Diäten ausgeglichen, so dass die Diäten jeweils die gleichen Konzentrationen an α-TÄ aufwiesen.

Vitamin E gilt als das wichtigste lipidlösliche Antioxidans in Geweben, Erythrozyten und Plasma, dessen Hauptfunktion der Schutz von Membranlipiden, Lipoproteinen und Depotfetten vor oxidativen Schädigungen ist (BURTON und INGOLD 1986, BURTON und TRABER 1990). Es hemmt sehr effektiv die Lipidperoxidation, indem die phenolische Hydroxygruppe am Chromanring mit organischen Peroxylradikalen reagiert und dadurch einen Kettenabbruch herbeiführt (BRIGELIUS-FLOHÉ und TRABER 1999, BURTON und INGOLD 1986, BUTTRISS und DIPLOCK 1988, ESTERBAUER et al. 1991, SIES 1989). *In*

*vitro*-Untersuchungen deuteten bereits an, dass der Cholesterolanteil in Lipoproteinen und Membranen sekundär als Folge der Peroxidation der PUFAs oxidiert wird (MUTO et al. 1983, SEVANIAN und MCLEOD 1987, TERAO et al. 1985,). Auch die eigenen Untersuchungen zur Wirkung von PUFA-reichem Lachsöl auf die Bildung bestimmter Oxysterole unterstützen diesen Befund (BRANDSCH et al. 2002, RINGSEIS und EDER 2002 und 2003).

Die Ergebnisse aus Studie 3 zeigten, dass Vitamin E offensichtlich nicht nur die Peroxidation von PUFAs, sondern auch die Bildung nicht-enzymatisch gebildeter Oxysterole wie 7β-OH-Chol *in vivo* hemmt. Die Beobachtung in Studie 3, dass bei Aufnahme von Kokosfett im Gegensatz zu Fischöl die Vitamin E-Zulagen keinen Einfluss auf die 7β-OH-Chol-Konzentrationen in den untersuchten Geweben hatten, lässt sich vermutlich damit erklären, dass Kokosfett ähnlich wie Schweineschmalz wenig oxidationssensitive n-3 PUFA enthält und damit weder die endogene Lipidperoxidation noch den postabsorptiven Vitamin E-Verbrauch in nennenswertem Umfang erhöht (IBRAHIM et al. 1997). Anhand des errechneten Vitamin E-Minimalbedarfs nach MUGGLI (1994) für die beiden Diätfette ergab sich, dass selbst die niedrigste Vitamin E-Konzentration von 10 mg α-TÄ/kg Diät ausreichend war, die im Kokosfett enthaltenen PUFAs vor Peroxidation zu schützen. Demgegenüber reichte bei Verwendung von Lachsöl als Diätfett erst eine Vitamin E-Konzentration von 40 mg α-TÄ näherungsweise aus, den Vitamin E-Minimalbedarf nach Muggli zu decken. Da die Polyenfettsäuren im Lachsöl bei den beiden niedrigen Vitamin E-Zulagen (10 und 20 mg α-TÄ) nicht ausreichend vor Peroxidation geschützt waren, kam es als Folge einer erhöhten Lipidperoxidation auch zu erhöhten 7β-OH-Chol-Konzentrationen. Ohne ausreichenden antioxidativen Schutz führt eine hohe Zufuhr an Polyenfettsäuren zu einem Vitamin E-Mangel (MACKENZIE et al. 1941), so dass anzunehmen ist, dass die Lachsölgefütterten Tiere, die 10 bzw. 20 mg α-TÄ/kg Diät erhielten, einen Vitamin E-Mangel aufwiesen. Darauf deuten insbesondere die reduzierte Futteraufnahme und Lebendmassezunahme dieser Tiere hin (RINGSEIS und EDER 2002). Die Wachstumsdepression im Vitamin E-Mangel ist wahrscheinlich Folge der erhöhten Peroxidation der PUFAs, welche zu Beeinträchtigungen der Membranfunktionen, des Muskelstoffwechsels und des Nervensystems führen (WITTING und HORWITT 1964). Initiiert wird die Lipidperoxidation im Vitamin E-Mangel vermutlich durch eine starke $H_2O_2$-Bildung in den Mitochondrien (CHOW et al. 1999), welches (via Haber-Weiss-Reaktion) zu hochreaktiven Hydroxylradikalen weiterreagieren kann. Dies

erklärt auch, dass die Fischöl-induzierte Depletierung der Vitamin E-Speicher zu einer starken $H_2O_2$-Bildung führt (ATALAY et al. 2000).

Die Ergebnisse aus Studie 3 lassen den Schluss zu, dass bei einer ausreichenden Vitamin E-Versorgung die Verabreichung eines PUFA-reichen Fettes im Vergleich zu einem PUFA-armen Fett die 7β-OH-Chol-Konzentration in LDL, Plasma und Geweben mit Ausnahme der Leber nicht beeinflusst. Dies deutet daraufhin, dass die Deckung des Vitamin E-Minimalbedarfes nicht ausreichend ist, die hepatischen Lipidstrukturen vor dem Fischöl-induzierten Anstieg der Lipidperoxidation zu schützen. Obwohl Vitamin E die TBARS-Bildung hemmt (BUCKINGHAM 1985, FLADER et al. 2003, HARATS et al. 1991, IBRAHIM et al. 1997, SUAREZ et al. 1999), war deren Konzentration in der Leber selbst bei einer sehr hohen Vitamin E-Aufnahme von 400 mg α-TÄ/kg Diät (Studie 1) bei Fütterung von Lachsöl signifikant höher als bei Fütterung von Schweineschmalz (BRANDSCH et al. 2002). Der enorm erhöhte Vitamin E-Bedarf der Leber bei Aufnahme von Fischöl erklärt sich vermutlich daraus, dass die Leber das Hauptzielorgan für oxidativen Stress ist. Denn die Inkorporierung der n-3-PUFA erhöht die Peroxidationsneigung der Leberlipide, was sich an den infolge Fischölfütterung stark erhöhten TBARS-Konzentrationen zeigt (DRAPER et al. 1986, IBRAHIM et al. 1997, KINALSKI et al. 1999, SAITO und NAKATSUGAWA 1994, SONG et al. 2000, STANGL et al. 2000). Zusätzlich steigern die oxidationssensitiven n-3 PUFA über die Induzierung der peroxisomalen β-Oxidation die Lipidperoxidation in der Leber (siehe Punkt 4.1.1).

Insgesamt zeigen die Ergebnisse aus den Studien 2 und 3 der vorliegenden Arbeit, dass der Effekt der Vitamin E-Konzentration auf die 7β-OH-Chol-Konzentration entscheidend von der Art des Fettes abhängt. Bei Aufnahme eines PUFA-reichen Fettes führt eine zu niedrige Vitamin E-Versorgung zu einem starken Vitamin E-Mangel, der sich in einem starken Anstieg der Lipidperoxidation und sekundär in einem Konzentrationsanstieg an 7β-OH-Chol bemerkbar macht (RINGSEIS und EDER 2002). Da Human- und Tierstudien bezüglich der Wirkung einer Vitamin E-Supplementierung auf die Oxysterolkonzentrationen im Organismus ein sehr uneinheitliches Bild ergaben (PORKKALA-SARATAHO et al. 2000, MOL et al. 1997, IULIANO et al. 2001, UPSTON et al. 2001, WISEMAN et al. 1995), ist zu vermuten, dass hierfür ein unterschiedlicher Vitamin E-Status bzw. ein erhöhter Vitamin E-Bedarf zwischen den verschiedenen Studienkollektiven vor der Supplementierungsphase ursächlich ist. So ist die Verringerung der Oxysterolkonzentrationen durch eine α-

Tocopherol-Supplementierung in der Studie von UPSTON et al. (2001) vermutlich darauf zurückzuführen, dass die Tiere vor der Supplementierung eine Vitamin E-defizitäre Diät erhielten und somit wahrscheinlich einen Vitamin E-Mangel aufwiesen. Auch in zwei Humanstudien ist möglicherweise ein suboptimaler Vitamin E-Status zu Beginn der Supplementierungsphase für die Senkung der Oxysterolkonzentrationen nach der Vitamin E-Supplementierung verantwortlich (PORKKALA-SARATAHO et al. 2000, MOL et al. 1997). Beispielsweise bewirkte die Vitamin E-Supplementierung bei Diabetikern (MOL et al. 1997), nicht jedoch bei gesunden Probanden (IULIANO et al. 2001, MOL et al. 1997), eine Senkung der Oxysterol- (7β-OH-Chol, 7-Keto-Chol, Cholestanetriol) und TBARS-Konzentrationen. Allerdings wiesen die Diabetiker in der Studie von MOL et al. (1997) zu Beginn der Supplementierungsphase gegenüber den gesunden Kontrollprobanden erhöhte TBARS-Konzentrationen auf. Oxidativer Stress und eine erhöhte Oxidierbarkeit des LDL im Diabetes mellitus sind mehrfach in der Literatur beschrieben (GRIESMACHER et al. 1995, HUNT et al. 1990, LYONS 1991). Dies lässt deshalb vermuten, dass der Vitamin E-Status der Diabetiker durch die Vitamin E-Supplementierung verbessert wurde und damit oxidativer Stress und die Oxysterolbildung vermindert wurden. In der Studie von IULIANO et al. (2001) führte Vitamin E wie bei den gesunden Probanden in der Studie von MOL et al. (1997) zu keiner signifikanten Verringerung an 7β-OH-Chol und 7-Keto-Chol im Plasma. Da bei diesen Probanden die Vitamin E-Supplementierung nur zu einer geringen Erhöhung der Plasma-Vitamin E-Konzentration führte, ist anzunehmen, dass der Vitamin E-Status vor der Intervention bereits bedarfsdeckend war.

### 4.1.4   Effekt einer Zufuhr von oxidiertem Cholesterin

Die Oxidation von Cholesterin in Lebensmitteln durch Einwirkung von Luftsauerstoff, Licht, Temperatur und lange Lagerdauer führt zur Bildung von Oxysterolen (YAN et al. 1999). Untersuchungen der Oxysterolkonzentrationen in Lebensmitteln führten zur Schätzung, dass etwa 1% des in der Nahrung enthaltenen Cholesterins in oxidierter Form aufgenommen wird (VAN DE BOVENKAMP et al. 1988). Daher sind Studien, die die Wirkung von nahezu vollständig oxidiertem Cholesterin im Organismus untersuchen, unphysiologisch und im Hinblick auf die Humanernährung wenig aussagekräftig. In den Studien 2, 4 und 5 wurde deshalb als Oxysterolquelle ein Cholesterinpräparat verwendet, das relativ moderat oxidiert war und damit besser die Situation in der Humanernährung reflektiert.

Wie in Studie 2 zu beobachten war, führte die Verabreichung von oxidiertem Cholesterol zu einem Konzentrationsanstieg an 7β-OH-Chol in den untersuchten Geweben. Studie 4 demonstrierte darüber hinaus, dass auch die Konzentrationen an 7-Keto-Chol in der Leber durch oxidiertes Cholesterol erhöht wurden. Dies stimmt mit Beobachtungen aus anderen Studien überein (OSADA et al. 1995 und 1998, VINE et al. 1998) und ist größtenteils auf die Absorption der Oxysterole im Darm und deren Transport in Chylomikronen zur Leber zurückzuführen (EMANUEL et al. 1991, LINSEISEN und WOLFRAM 1998a, VINE et al. 1997). Erhöhte Oxysterolkonzentrationen in der VLDL-Fraktion nach Verabreichung oxidierten Cholesterins (VINE et al. 1998) sind ein Indiz dafür, dass exogene Oxysterole auch aus der Leber in die Zirkulation ausgeschleust werden. Auf eine hepatische Sekretion von Oxysterolen deuten auch die bei Fütterung oxidierten Cholesterins gegenüber reinem Cholesterin erhöhten 7β-OH-Chol-Konzentrationen in der LDL-Fraktion in Studie 2 der vorliegenden Arbeit hin, da die LDL-Partikel während der Zirkulation im Blut aus den VLDL-Partikeln durch Einwirkung der endothelialen Lipoproteinlipase hervorgehen. Es ist deshalb davon auszugehen, dass die signifikant erhöhten Plasmakonzentrationen an 7β-OH-Chol vor allem auf einen Konzentrationsanstieg in der VLDL- und LDL-Fraktion zurückzuführen sein dürften.

Möglicherweise ist der Anstieg der Konzentrationen an 7β-OH-Chol aber auch zu einem geringen Teil darauf zurückzuführen, dass Oxysterole vermutlich per se prooxidativ wirken, d.h. oxidativen Stress erzeugen und in der Folge die Lipidperoxidation initiieren. Unter anderem deutet die Depletierung endogener Antioxidantien auf oxidativen Stress hin (FREI et al. 1988). BANSAL et al. (2001) konnten nachweisen, dass die Inkubation von Macrophagen mit 7β-OH-Chol zu verminderten Konzentrationen an reduziertem GSH führt (BANSAL et al. 2001). Verminderte Konzentrationen an GSH (reduziert und gesamt) wurden auch in Studie 4 der vorliegenden Arbeit als Folge der Verabreichung von oxidiertem Cholesterin beobachtet, und sind Anzeichen für einen erhöhten Verbrauch an GSH infolge von oxidativem Stress (ALEYNIK et al. 1999, DE und DARAD 1988, KINALSKI et al. 1999, SARKAR et al. 1995). Auch die Beobachtung, dass die Bildung von Oxysterolen in Synaptosomen und/oder Mitochondrien zu einer Reduzierung der Konzentrationen an α-Tocopherol führt (VATASSERY et al. 1997a und 1997b), ist ein Hinweis auf die Induzierung von oxidativem Stress durch Oxysterole. Eine Reduzierung der α-Tocopherolkonzentrationen in Plasma und LDL nach Fütterung oxidierten Cholesterins im Vergleich zu reinem Cholesterin konnte auch

in Studie 2 (Exp. 2) nachgewiesen werden. Allerdings war dieser Effekt nur bei den Lachsöl-supplementierten Tieren, nicht jedoch bei den Kokosfett-supplementierten Tieren zu beobachten. Dies erklärt sich vermutlich dadurch, dass die infolge der Fischölfütterung stark erhöhte Peroxidationsneigung der Lipidstrukturen in Verbindung mit dem mutmaßlich prooxidativ wirksamen oxidierten Cholesterin den Vitamin E-Verbrauch zusätzlich erhöht. In verschiedenen Zelltypen wird in Gegenwart von 7β-OH-Chol und 7-Keto-Chol Apoptose beobachtet (LIZARD et al. 1996, 1998 und 2000, LYONS et al. 2001, O´CALLAGHAN et al. 1999). Auch dies wird als Indiz für die Induzierung von oxidativem Stress und der Bildung von ROS gewertet, da diese Apoptose durch Glutathion und α-Tocopherol verhindert werden konnte (LIZARD et al. 1998 und 2000, LYONS et al. 2001). Hinweise auf eine gesteigerte Lipidperoxidation durch Oxysterole bzw. oxidiertes Cholesterin stammen sowohl aus *in vivo* als auch *in vitro* Untersuchungen. Bespielsweise ergaben tierexperimentelle Studien, dass oxidiertes Cholesterin im Vergleich zu reinem Cholesterin zu einer Erhöhung der TBARS-Konzentrationen in Plasma und Leber führt (OSADA et al. 1995 und 1998). Ursächlich hierfür ist möglicherweise, dass absorbierte und inkorporierte Oxysterole die Bildung freier Radikale induzieren, welche durch Reaktion mit polyungesättigten Fettsäuren zur Bildung von TBARS führen (OSADA et al. 1998). Auch in Zellkulturen führte die Inkubation mit spezifischen Oxysterolen zu einem Anstieg der Lipidperoxidation (TBARS), welche durch Zugabe von α-Tocopherol oder Butyliertem Hydroxytoluen vermindert werden konnte (WILSON et al. 1997). In Rattenhepatozyten, die mit 7-Keto-Chol oder Cholestanetriol inkubiert wurden, bewirkte eine kommerzielle Tocopherolmischung (Covi-ox) eine Reduzierung der TBARS-Konzentrationen, was ebenfalls auf einen prooxidativen Effekt von Oxysterolen hinweist (CANTWELL und DEVERY 1998). In der Studie von LYONS et al. (2001) führte 7β-OH-Chol zu keinem Anstieg der TBARS-Konzentrationen, allerdings deutet die Inhibierung der Oxysterol-induzierten Apotose auch hier auf die Beteiligung von ROS hin. Insgesamt deuten die Ergebnisse aus *in vitro* und *in vivo* Studien deshalb daraufhin, dass Oxysterole per se prooxidativ wirken. Da jedoch in den Studien 2 und 4 der vorliegenden Arbeit lediglich Parameter des antioxidativen Schutzsystems, nicht aber die Konzentrationen an TBARS gemessen wurden, kann nicht definitiv beurteilt werden, ob der Oxysterolanstieg bei Fütterung oxidierten Cholesterins im Vergleich zu reinem Cholesterin in Studie 2 auch auf einen prooxidativen Effekt der Oxysterole zurückzuführen ist.

Es konnte gezeigt werden, dass oxidiertes Cholesterin, welches signifikante Mengen an 7β-OH-Chol und anderen Oxysterolen enthält, zu einem Konzentrationsanstieg an 7β-OH-Chol

in Blut und Leber von Ratten führt, was in erster Linie auf dessen Absorption im Darm und dessen Transport zur Leber zurückzuführen sein dürfte. Darüberhinaus zeigte sich, dass dies auch zu erhöhten 7β-OH-Chol-Konzentrationen in Plasma, LDL und Erythrozyten führt. Insbesondere die Erhöhung der Konzentrationen an 7β-OH-Chol in LDL und Plasma sind im Hinblick auf das damit verbundene erhöhte Arterioskleroserisiko (SALONEN et al. 1997, ZIEDEN et al. 1999) als kritisch anzusehen.

## 4.2    Physiologische Wirkungen oxidierten Cholesterins

## 4.2.1 Wirkung einer Zufuhr oxidierten Cholesterins auf das antioxidative Schutzsystem

*In vitro*-Studien lieferten bereits Hinweise, dass Oxysterole wie 7β-OH-Chol und 7-Keto-Chol Parameter des antioxidativen Schutzsystems modulieren und oxidativen Stress induzieren (BANSAL et al. 2001, CANTWELL und DEVERY 1998, LIZARD et al. 2000, LYONS et al. 2001, MCCLUSKEY et al. 1999, O´CALLAGHAN et al. 2002). Allerdings liegen bislang nur wenige Studien vor, die die Wirkung oxidierten Cholesterins auf das antioxidative Schutzsystem *in vivo* untersuchen. So lieferten vergleichende Studien zwischen oxidiertem und reinem Cholesterin Hinweise darauf, dass oxidiertes Cholesterin möglicherweise die Lipidperoxidation in der Leber von Ratten steigert (OSADA et al. 1995, 1998 und 1999), was auf die Induzierung von oxidativem Stress schliessen lässt. Zur Beurteilung der Lipidperoxidation wurden allerdings in den Studien von OSADA et al. (1995, 1998 und 1999) die Messung der TBARS herangezogen, die lediglich einen Aufschluss über die Peroxidationsneigung des untersuchten Gewebes *in vitro* ergibt (BUCKINGHAM 1985, STANGL et al. 2000). Da im Organismus die Lipidperoxidation erst dann auftritt, wenn das antioxidative Schutzsystem den induzierten oxidativen Stress nicht bewältigen kann, wurde in Studie 4 untersucht, ob auch Parameter des antioxidativen Schutzsystems durch oxidiertes Cholesterin moduliert werden.

Insgesamt zeigte sich in Studie 4 der vorliegenden Arbeit, dass oxidiertes Cholesterin oxidativen Stress auch *in vivo* in der Leber von Ratten hervorruft. Dies zeigte sich unter anderem in einer erhöhten Expression und Aktivität antioxidativer Enzyme und einer verminderten Konzentration an Gesamt-GSH und reduziertem GSH. Dies sind Effekte, die mehrfach als Konsequenz von oxidativem Stress in der Leber von Ratten bzw. Mäusen

beobachtet wurden (FLADER et al. 2003, IBRAHIM et al. 1997, SHULL et al. 1991). Wider Erwarten war der Effekt des oxidierten Cholesterins auf das antioxidative Schutzsystem bei Fütterung von Kokosfett genauso ausgeprägt wie bei Fütterung von Lachsöl, obwohl die Fütterung von Fischöl die Peroxidationsempfindlichkeit der Membranen stark erhöht und oxidativen Stress induziert (CHO und CHOI 1994, IBRAHIM et al. 1997, JAVOUHEY-DONZEL et al. 1993, KUBOW et al. 1996). Dies zeigt, dass das antioxidative Schutzsystem auch bei niedriger Peroxidationsneigung der Gewebe, wie bei Fütterung von Kokosfett, durch oxidiertes Cholesterin bereits massiv gestresst wird und dieser oxidative Stress durch den prooxidativen Effekt des Fischöls und den erhöhten Konzentration an 7β-OH-Chol und 7-Keto-Chol nicht weiter verstärkt wird. Möglicherweise deutet dies auf einen Sättigungseffekt in der Expression und Aktivität antioxidativer Enzyme hin, der auch in ähnlicher Weise in einer anderen Untersuchung beobachtet wurde (ENOMOTO et al. 2001).

Insgesamt decken sich die Befunde aus Studie 4 mit der Beobachtung aus *in vitro*-Studien, dass Oxysterole wie 7β-OH-Chol und 7-Keto-Chol oxidativen Stress auf zellulärer Ebene erzeugen (BANSAL et al. 2001, LIZARD et al. 1998 und 2000, LYONS et al. 2001). Unter anderem wurde *in vitro* ein Anstieg diverser antioxidativer Enzymaktivitäten und eine Verminderung der GSH-Konzentrationen in humanen Monozyten und Macrophagen und Rattenhepatozyten festgestellt (BANSAL et al. 2001, CANTWELL und DEVERY 1998, LYONS et al. 2001, O´CALLAGHAN et al. 2002). Ursächlich hierfür ist vermutlich ein starker Anfall von Superoxidanionradikalen, der im Zellsystem bei Inkubation mit 7β-OH-Chol und 7-Keto-Chol, nicht jedoch mit 7α-OH-Chol, beobachtet wurde (MIGUET-ALFONSI et al. 2002). Da Superoxidanionen die Bildung weiterer ROS fördern und damit die Lipidperoxidation initiieren können, wird der steile 7-Keto-Chol-induzierte Anstieg der Aktivität der Superoxiddismutase (SOD), welche die Dismutationsreaktion der Superoxidanionenradikale katalysiert (MARKLUND 1984), als ein Schutzmechanismus der Zelle vor oxidativen Schädigungen interpretiert (CANTWELL und DEVERY 1998). Auch die erhöhte Expression der cytosolischen Cu/Zn-SOD in der Leber nach Aufnahme oxidierten Cholesterins in Studie 4 der vorliegenden Arbeit ist deshalb ein Hinweis auf einen starken Anfall von Superoxidanionen und auf eine Stimulierung des antioxidativen Schutzsystems durch exogene Oxysterole. Das aus der SOD-katalysierten Dismutationsreaktion hervorgehende $H_2O_2$ wird durch die im Cytosol und in der mitochondrialen Matrix lokalisierte Glutathionperoxidase detoxifiziert (BIESALSKI und FRANK 1995, O`BRIEN 1988), welche ebenfalls in der Studie 4 sowohl in ihrer Aktivität als auch in ihrer

Genexpression durch oxidiertes Cholesterin erhöht war. Da GSH durch die Aktivität der GSH-Px verbraucht wird, erklärt dies auch die Depletierung von GSH, die in Studie 4 der vorliegenden Arbeit und mehrfach bei der Oxysterol-induzierten Apoptose an Zellen der Arterienwand wie Endothelzellen und glatten Muskelzellen und an zirkulierenden Blutzellen wie Monozyten und Macrophagen nachgewiesen wurde (BANSAL et al. 2001, LIZARD et al. 1997 und 1998, O´CALLAGHAN et al. 2002).

MIGUET-ALFONSI et al. (2002) sehen die Apoptose-Induzierung und die Stimulierung der Superoxidanionenbildung und der Lipidperoxidation in den Zellen der Arterienwand als Ursache für die gefäßschädigende und proatherogene Wirkung von 7β-OH-Chol und 7-Keto-Chol. Die Beobachtung in Studie 4 der vorliegenden Arbeit, dass die Fütterung oxidierten Cholesterins oxidativen Stress erzeugt und das antioxidative Schutzsystem stresst, liefert daher möglicherweise auch einen Erklärungsansatz dafür, dass oral oder intravenös verabreichtes oxidiertes Cholesterin, das pathophysiologisch bedeutsame Oxysterole enthält, in mehreren Studien verschiedene proatherogene Wirkungen wie Schaumzellbildung, endotheliale Dysfunktion und Cholesterolakkumulierung in der Arterienwand zeigte (RONG et al. 1998 und 1999, STAPRANS et al. 1998, VINE et al. 1998).

## 4.2.2 Wirkung einer Zufuhr oxidierten Cholesterins auf Parameter des Lipidstoffwechsels und der VLDL-Synthese und –Sekretion

Vergleichende Studien zwischen reinem und oxidiertem Cholesterin ergaben, dass oxidiertes Cholesterin den Lipidstoffwechsel von Ratten und Kaninchen beeinflusst (OSADA et al. 1994b, 1995, 1996 und 1998, VINE et al. 1998). Unter anderem zeigte sich, dass oxidiertes gegenüber reinem Cholesterin zu einer Beeinflussung des Fettsäure- und Cholesterinmetabolismus in der Leber führt (OSADA et al. 1998 und 1999, SMITH und JOHNSON 1989, VARGAS et al. 1986). Dies ist vermutlich auf die biologische Wirkung der Oxysterole auf zellulärer Ebene zurückzuführen (GUARDIOLA et al. 1996, MOREL und LIN 1996), da Oxysterole, wie auch in Studie 4 der vorliegenden Arbeit gezeigt, aus dem Darm absorbiert und via Chylomikronen zur Leber transportiert werden (OSADA et al. 1994a, VINE et al. 1997).

Erhöhte Anteile an Arachidonsäure in den Lipidfraktionen Triglyzeride, Phosphatidylethanolamin und Phosphatidylcholin wurden ebenfalls in Studie 5 der vorliegenden Arbeit beobachtet. Dies deutet auf eine erhöhte Desaturierung der Linolsäure durch die mikrosomale Δ6-Desaturase, des geschwindkeitsbestimmenden Enzyms der Bildung von Arachidonsäure, hin und wird auch durch andere Untersuchungen gestützt, die eine erhöhte Δ6-Desaturaseaktivität bei Fütterung oxidierten Cholesterins im Vergleich zu reinem Cholesterin nachweisen konnten (OSADA et al. 1998 und 1999). Wie OSADA et al. (1998) vermuteten, ist dies auf eine veränderte Fluidität der hepatischen Mikrosomenmembran zurückzuführen. Diese Vermutung wird auch dadurch unterstützt, dass BRENNER et al. (1981) zeigen konnten, dass die Aktivitäten der Fettsäuredesaturasen durch die Fluidität der Mikrosomenmembranen beeinflusst werden. Da die Inkorporierung von Oxysterolen in Membranen zu einer Beeinflussung der Membraneigenschaften wie Fluidität, Permeabilität und Stabilität führt (ROONEY et al. 1985, THEUNISSEN et al. 1986), ist deshalb zu vermuten, dass dies auch die Ursache für die gesteigerte Desaturierung der Linolsäure in Studie 5 der vorliegenden Arbeit ist. Möglicherweise ist diese gesteigerte Desaturierung der Linolsäure als ein Kompensationsmechanismus für eine veränderte Membranfluidität aufzufassen, so wie auch eine erhöhte Aktivität der Stearoyl-CoA-Desaturase bei Fütterung von Cholesterin als ein Kompensationsmechanismus interpretiert wird (BRENNER 1990, GARG et al. 1986, SIVARAMAKRISHNAN und PYNADATH 1982). Membranständiges Cholesterol verringert die Fluidität der Membranen, indem es die Fettsäuren der Phospholipide immobilisiert („Condensing-Effekt") (STILLWELL et al. 1994). Oxysterole verstärken vermutlich diesen Condensing-Effekt (THEUNISSEN et al. 1986), worauf die Beobachtung hindeutet, dass die Inkorporierung von 7α-OH-Chol in die Membran in einer Menge von 10% des Gesamtsterolgehaltes den gleichen Effekt auf die Immobilisierung der Fettsäureketten aufweist wie 50% Cholesterol (ROONEY et al. 1985). In der Erythrozytenmembran bewirkt diese Oxysterol-Inkorporierung eine verringerte Hämolyseempfindlichkeit der Erythrozyten (STREULI et al. 1981). Dies konnte auch mittels eines *in vitro*-Hämolysetests nach der Methode von O´DELL et al. (1987) – ein Testverfahren zur Beurteilung der Stabilität der Erythrozytenmembran (BUCKINGHAM 1985, KIRCHGESSNER et al. 1994) - bei den Erythrozyten der Ratten der Studie 2 der vorliegenden Arbeit nachgewiesen werden (Abbildung 2; unveröffentlichte Daten). So zeigte sich bei der höchsten NaCl-Konzentration, dass die Erythrozyten der Tiere, die oxidiertes Cholesterin erhielten, eine signifikant erhöhte Hämolyseresistenz bzw. eine erniedrigte Hämolyseempfindlichkeit aufwiesen gegenüber den Erythrozyten der Tiere, die reines

Cholesterin erhielten. Die beobachtete verminderte Hämolyseresistenz infolge der Verabreichung von Lachsöl wird auf die starke Inkorporierung der oxidationsempfindlichen n-3-PUFA in Erythrozytenmembran zurückgeführt (HAGVE et al. 1993, KIRCHGESSNER et al. 1994, PALOZZA et al. 1996).
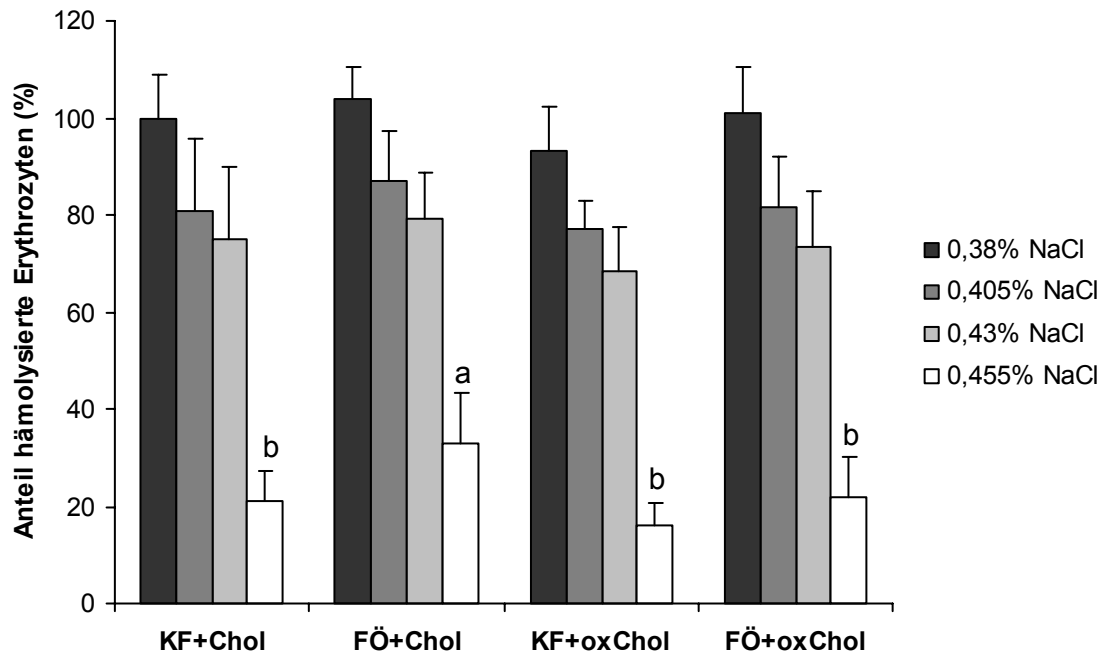


**Abb. 2:**         Hämolyseresistenz der Erythrozyten in hypotonen Natriumchloridlösungen der Ratten aus Studie 5.
Die Balken stellen Mittelwert ± Standardabweichung für 9 Tiere pro Gruppe dar. Unterschiedliche hochgestellte Kleinbuchstaben markieren signifikante Unterschiede zwischen den Gruppen innerhalb einer NaCl-Konzentration im Mittelwertsvergleich mit Fisher-Test. Signifikanz der Faktoren: Fett ($P<0,05$), Cholesterol ($P<0,05$).

Die hepatischen Lipidkonzentrationen wurden in Studie 5 durch oxidiertes Cholesterin nicht beeinflusst. Dies steht im Gegensatz zu den Ergebnissen anderer Studien, bei denen insbesondere die Cholesterinkonzentrationen durch oxidiertes Cholesterin vermindert wurden (OSADA et al. 1994b, 1995 und 1998). Möglicherweise liegt dies daran, dass in den Untersuchungen von OSADA et al. (1994b, 1995 und 1998) die hohe Oxysterolkonzentration in dem stark oxidierten Cholesterin zu der mehrfach beschriebenen Hemmung der hepatischen HMG-CoA-Reduktase durch Oxysterole führte (ERICKSON et al. 1977, SAUCIER et al. 1993, VARGAS et al. 1986), während dies in der eigenen Studie aufgrund der Verwendung eines moderat oxidierten Cholesterins mit sehr hohem Gehalt an unoxidiertem Cholesterol nicht der Fall war. Möglicherweise wurde aber auch der Effekt der Oxysterole auf die Hemmung der Cholesterinbiosynthese in der Studie 5 der vorliegenden Arbeit durch die hohe Aufnahme an unoxidiertem Cholesterin überlagert.

In Übereinstimmung mit anderen Studien zeigte die eigene Untersuchung (Studie 5), dass die Fütterung oxidierten gegenüber reinen Cholesterins zu verminderten Triglycerid- und/oder Cholesterolkonzentrationen im Plasma führt (CHO et al. 1986, OSADA et al. 1994b und 1999, VINE et al. 1998). Darüberhinaus zeigte sich in Studie 5, dass oxidiertes Cholesterin die Cholesterin- und Triglyceridkonzentrationen in der VLDL-Fraktion reduziert, was auf eine verminderte VLDL-Synthese und –Sekretion hindeutet. Wie anhand der Studie 5 beobachtet werden konnte, ist hierfür möglicherweise eine verminderte Transkription des MTP-Gens verantwortlich, da andere Parameter, welche ebenfalls die hepatische Lipoproteinsynthese beeinflussen (hepatische Lipidkonzentrationen, Genexpression des apoB) durch oxidiertes Cholesterin nicht beeinflusst wurden. Das MTP spielt eine entscheidende Rolle in der Synthese und Sekretion von VLDL in der Leber und von Chylomikronen im Darm, indem es den Transfer von Triglyceriden, Cholesterin, Cholesterinestern und Phospholipiden auf neu synthetisiertes apoB im endoplasmatischen Retikulum von Hepatozyten und Enterozyten bewerkstelligt (GREGG und WETTERAU 1994, JAMIL et al. 1995, WETTERAU und ZILVERSMIT 1986). Ein genetischer Defekt im MTP-Gen wie bei der autosomal rezessiven Erkrankung Abetalipoproteinämie führt deshalb zu einer massiven Störung der VLDL- und Chylomikronensynthese und zu stark reduzierten Plasmalipidkonzentrationen (REHBERG et al. 1996, RICCI et al. 1995, SHARP et al. 1993, WETTERAU et al. 1992).

Es konnte nachgewiesen werden, dass die Expression des MTP-Gens durch die Nahrung beeinflusst wird. So führen fettreiche Diäten bei Hamstern zu einer Erhöhung der hepatischen MTP mRNA-Konzentrationen (BENNETT et al. 1995). Auch die Fütterung einer Cholesterol-reichen Diät bewirkt bei Hamstern einen Anstieg der MTP mRNA-Konzentrationen (BENNETT et al. 1996), was über ein *Sterol Responsive Element* (SRE) im Promotorbereich des MTP-Gens vermittelt wird (HAGAN et al. 1994). Bislang gibt es keine Untersuchungen, die den Effekt von oxidiertem Cholesterol auf die MTP-Genexpression *in vivo* untersuchen. Allerdings ist eine Beeinflussung der Transkription des MTP-Gens durch Oxysterole anzunehmen, da zum einen verschiedene Oxysterole als Regulatoren des Lipidmetabolismus via Aktivierung des nukleären Transkriptionsfaktors *Liver X Receptor α* (LXRα) diskutiert werden (PEET et al. 1998) und zum anderen Gene, die auf transkriptionaler Ebene durch Oxysterole beeinflusst werden, über ein SRE in ihrem Promotor verfügen, das über ein SRE-Bindungsprotein (SREBP) kontrolliert wird (EDWARDS und ERICSSON

1998). Unterstützt wird diese Vermutung auch dadurch, dass gezeigt werden konnte, dass Zielgene des LXRα wie SREBP-1 und -2 zumindest *in vitro* an das SRE im MTP-Promotor binden (SATO et al. 1999). Dies würde auch erklären, weshalb das SREBP-1c (die Hauptisoform des SREBP-1 in der tierischen Leber) nicht nur die hepatische Lipogenese durch Aktivierung der Hauptgene der Fettsäuresynthese (HORTON et al. 2002), sondern auch die Synthese und Sekretion apoB-enthaltender Lipoproteine kontrolliert (WANG et al. 1997) und damit die VLDL-Synthese direkt beeinflusst.

In Studie 5 der vorliegenden Arbeit wurde spekuliert, dass eine verminderte Transkription der CYP7A1 für die reduzierten MTP mRNA-Konzentrationen und damit für eine verminderte VLDL-Sekretion in Studie 5 verantwortlich ist. Es konnte nämlich mehrfach nachgewiesen werden, dass oxidiertes Cholesterin die Aktivität der CYP7A1 im Vergleich zu reinem Cholesterin vermindert (BÖSTROM 1983, OSADA et al. 1994b, 1995 und 1998) und die Genexpression der CYP7A1 stark mit seiner Aktivität korreliert (NOSHIRO et al. 1990). Außerdem wurde gezeigt, dass zumindest *in vitro* eine positive Korrelation zwischen den mRNA-Konzentrationen der CYP7A1 und des MTP besteht, was auch der Grund dafür ist, dass die Gentranskription der CYP7A1 die hepatische Lipoproteinsynthese und -sekretion beeinflusst (WANG et al. 1997). Da die Aktivität oder Genexpression der CYP7A1 allerdings in der vorliegenden Arbeit nicht gemessen wurde, ist aufgrund der vorliegenden Datenlage und den bisherigen Befunden aus der Literatur keine gesicherte Aussage bezüglich der Wirkung von oxidiertem Cholesterin auf die MTP-Gentranskription zu treffen.

Mehrere Untersuchungen ergaben auch, dass der LDL-Rezeptor die VLDL-Produktion moduliert. So führte eine Up-Regulierung des LDL-Rezeptors und eine Erhöhung der hepatischen LDL-Aufnahme durch HMG-CoA-Reduktase-Inhibitoren wie den Statinen zu einer reduzierten VLDL-Produktion und -Sekretion (CUCHEL et al. 1997, GINSBERG et al. 1987, TWISK et al. 2000). Oxysterole wie 7-Ketocholesterol, 25-Hydroxycholesterol, Cholesterolepoxide und Cholestanetriol, die in den verwendeten Oxysterol-Diäten enthalten waren, hemmen dagegen zumindest *in vitro* die Genexpression des LDL-Rezeptors (PENG et al. 1991 und 1992). Die Gentranskription des LDL-Rezeptors wird durch Bindung des SREBP-1 an ein *sterol responsive element-1* aktiviert, das im Promotorbereich des LDL-Rezeptors liegt (BRIGGS et al. 1993, WANG et al. 1993). Oxysterole hemmen die LDL-Rezeptor-Gentranskription dadurch, dass sie die Abspaltung des in der Kernhülle bzw. im endoplasmatischen Retikulum verankerten aktiven SREBP-1-Vorläufers hemmen, aber auch

die Degradation des funktionalen SREBP-1-Proteins erhöhen (WANG et al. 1994). Da eine Hemmung der Transkription der HMG-CoA-Reduktase durch Oxysterole auch zu einer verminderten Transkription des LDL-Rezeptors führt (RENNERT et al. 1990, RIDGWAY 1995), ist nicht anzunehmen, dass die verminderten VLDL-Lipidkonzentrationen in Studie 5 der vorliegenden Arbeit in Zusammenhang mit einer Up-Regulierung des LDL-Rezeptors stehen. Dies stimmt auch mit der Beobachtung überein, dass oxidiertes Cholesterin *in vivo* zu einer Reduktion der HMG-CoA-Reduktase-Aktivität im Vergleich zu reinem Cholesterin führt (OSADA et al. 1994b, 1995 und 1998).

Die Studie 5 der vorliegenden Arbeit konnte zeigen, dass moderat oxidiertes Cholesterin *in vivo* Effekte aufweist, die auf eine Beeinflussung des Fettsäuremetabolismus und der Lipidkonzentrationen in Plasma und VLDL hinweisen. Die verminderten Plasma- und VLDL-Lipidkonzentrationen lassen in Verbindung mit der reduzierten MTP-Gentranskription vermuten, dass oxidiertes Cholesterin auch die VLDL-Synthese und –Sekretion moduliert. Die beobachtete erhöhte Desaturierung der Linolsäure ist vermutlich auf eine Veränderung der Fluidität der hepatischen Mikrosomenmembran aufgrund der Inkorporierung von Oxysterolen zurückzuführen. Durch diesen direkten Effekt auf die Membranstruktur werden nicht nur membranassoziierte Enzymsysteme wie die $\Delta$6-Desaturase, sondern vermutlich auch membranstänige Transportsysteme beeinflusst. Dies deuten einige *in vitro*-Studien an, die den Effekt spezifischer Oxysterole auf verschiedene Transportproteine wie $Na^+/K^+$-ATPase, $Ca^{2+}/Mg^{2+}$-ATPase und andere Transportmechanismen untersuchen (BOISSONNEAULT et al. 1991a und 1991b, BOISSONNEAULT und HEININGER 1985, PENG et al. 1985, WOOD et al. 1995, ZHOU et al. 1991). Die Oxysterol-induzierte Erhöhung des Albumintransfers in Endothelzellen (BOISSONNEAULT et al. 1991a und 1991b) und erhöhte intrazelluläre Calciumakkumulierung (ZHOU et al. 1991) deuten dabei auf proatherogene Wirkungen wie eine verminderte Barrierefunktion und eine erhöhte Permeabilität des Gefäßendothels hin, die möglicherweise mit den pathophysiologischen Wirkungen spezifischer Oxysterole in Verbindung stehen.

## 5.      Zusammenfassung

Untersuchungen ergaben, dass die Konzentrationen an „nicht-enzymatisch" gebildeten Oxysterolen wie 7β-Hydroxycholesterol (7β-OH-Chol) und 7-Ketocholesterol (7-Keto-Chol) geeignete Parameter zur Erfassung des *in vivo* Lipidperoxidationsgeschehens bzw. von oxidativem Stress sind. Da über die Diätzusammensetzung oxidativer Stress im Organismus erzeugt werden kann, sollte in der vorliegenden Arbeit im ersten Abschnitt der Frage nachgegangen werden, ob verschiedene Diätkomponenten bzw. Nährstoffe (Fischöl, Eisen, Vitamin E, oxidiertes Cholesterin) die Konzentrationen an „nicht-enzymatisch" gebildeten Oxysterolen bzw. 7β-OH-Chol beeinflussen. Die Ratte wurde als Versuchsobjekt verwendet, da sie ein geeignetes *in vivo*-Modell zur Induktion von oxidativem Stress und/oder zur Untersuchung der *in vivo*-Bildung von Oxysterolen darstellt. Zur Beantwortung der Fragestellung im ersten Abschnitt wurden drei Studien mit männlichen Ratten durchgeführt. In der ersten Studie wurden die Versuchsfaktoren Diätfett (Lachsöl vs. Schweineschmalz 100 g/kg Diät) und Eisensupplement (50 vs. 500 mg Eisensupplement/kg Diät) variiert. In der zweiten, zweigeteilten Studie wurden im Versuchsansatz 1 die Faktoren Diätfett (Fischöl vs. Kokosfett 100 g/kg Diät) und Vitamin E (40 vs. 240 mg α-Tocopheroläquivalente (α-TÄ)/kg Diät) und im Versuchsansatz 2 die Faktoren Cholesterin (reines Cholesterol vs. oxidiertes Cholesterol 5 g/kg Diät), Diätfett (Fischöl vs. Kokosfett 100 g/kg Diät) und Vitamin E (40 vs. 240 mg α-TÄ/kg Diät) variiert. In der dritten Studie wurden die Faktoren Diätfett (Fischöl vs. Kokosfett 100 g/kg Diät) und Vitamin E (10, 20, 40 vs. 240 mg α-TÄ/kg Diät) variiert. Die Untersuchungen des ersten Abschnitts ergaben bezüglich der untersuchten Oxysterolkonzentrationen folgende Ergebnisse:

*1      Effekt von Fischöl:* In Studie 1 waren die Konzentrationen aller gemessenen Oxysterole in der Leber, unabhängig von der Eisenkonzentration in der Diät, bei Fütterung von Lachsöl zwei- bis dreimal höher als bei Fütterung von Schweineschmalz. Die Konzentrationen an 7β-OH-Chol in Leber, Plasma, LDL und Erythrozyten in Studie 2 waren ebenfalls bei Fütterung von Lachsöl höher als bei Fütterung von Kokosfett - unabhängig von der Vitamin E-Konzentration in der Diät. In Studie 3 wiesen Ratten, die Fischöl als Diätfett erhielten, lediglich in der Leber, unabhängig von der Vitamin E-Versorgung, höhere Konzentrationen an 7β-OH-Chol auf als Ratten, die Kokosfett als Diätfett erhielten. In Plasma, LDL und Erythrozyten war der Effekt des Fischöls auf die 7β-OH-Chol-Konzentration dagegen abhängig von der Vitamin E-Versorgung. Nur die Lachsöl-supplementierten Tiere, die 10 und 20 mg α-TÄ/kg Diät erhielten, wiesen im Vergleich zu

den entsprechenden Kokosfett-supplementierten Tieren höhere Konzentrationen an 7β-OH-Chol auf.

**2**     *Effekt von Eisen:* Der Effekt der Eisenkonzentration in der Diät auf die Oxysterolkonzentrationen war abhängig vom Diätfett. Ratten, die Lachsöl als Diätfett und die hohe Eisenkonzentration erhielten, wiesen höhere Konzentrationen an 7β-OH-Chol, 7-Keto-Chol und Cholestanetriol in der Leber auf als Ratten, die Lachsöl und die niedrige Eisenkonzentration in der Diät erhielten. Bei den Ratten, die Schweinschmalz als Diätfett erhielten, wurden diese Oxysterolkonzentrationen durch die Höhe der Eisenkonzentration in der Diät dagegen nicht beeinflusst. Die Konzentrationen der Cholesterolepoxide wurden durch die Höhe der Eisenkonzentration in der Diät nicht beeinflusst.

**3**     *Effekt von Vitamin E:* In Studie 2 wurden die 7β-OH-Chol-Konzentrationen nur im Plasma, nicht jedoch in der Leber, LDL und Erythrozyten durch die Vitamin E-Konzentration in der Diät beeinflusst. Eine Erhöhung der Vitamin E-Konzentration in der Diät von 40 auf 240 mg α-TÄ/kg Diät führte sowohl bei Fütterung von Kokosfett als auch bei Fütterung von Lachsöl zu einer Reduzierung der 7β-OH-Chol-Konzentration im Plasma. In Studie 3 hatte dagegen die Vitamin E-Versorgung bei den Kokosfett-gefütterten Ratten keinen Einfluss auf die 7β-OH-Chol-Konzentration in allen untersuchten Proben. Bei Fütterung von Lachsöl wiesen die Tiere bei der niedrigsten Vitamin E-Versorgung (10 mg α-TÄ/kg Diät) die höchsten Konzentrationen an 7β-OH-Chol in allen untersuchten Proben auf. Eine kontinuierliche Steigerung der Vitamin E-Versorgung auf 40 mg α-TÄ/kg Diät bewirkte eine Senkung der Konzentrationen an 7β-OH-Chol in Leber, Plasma, LDL und Erythrozyten. Eine weitere Steigerung der Vitamin E-Versorgung auf 240 mg α-TÄ/kg Diät erbrachte jedoch keine weitere Absenkung.

**4**     *Effekt von oxidiertem Cholesterin:* Die Fütterung oxidierten Cholesterins in Studie 3 bewirkte bei den Kokosfett-supplementierten Ratten in allen untersuchten Proben einen Anstieg der Konzentrationen an 7β-OH-Chol, bei den Lachsöl-supplementierten Tieren jedoch nur in Leber und Plasma. Eine zusätzliche Erhöhung der Vitamin E-Konzentration in der Diät führte nur im Plasma zu einer Reduzierung der 7β-OH-Chol-Konzentrationen.

Als Fazit des ersten Untersuchungsabschnitts ergab sich, dass die Konzentrationen an „nicht-enzymatisch" gebildeten Oxysterolen bzw. 7β-OH-Chol bei Verabreichung eines n-3-PUFA-reichen Fettes, insbesondere bei niedriger Vitamin E-Versorgung, höher sind als bei einem n-3-PUFA-armen Fett. Eine hohe Zufuhr an Eisen führte nur in Verbindung mit einem n-3-PUFA-reichen Fett zu einem Anstieg der Konzentrationen an 7β-OH-Chol und anderer

„nicht-enzymatisch" gebildeter Oxysterole. Oxidiertes Cholesterin erhöhte unabhängig von der Fettart die 7β-OH-Chol-Konzentrationen.

In einem zweiten Abschnitt der vorliegenden Arbeit sollte der Frage nachgegangen werden, ob exogene Oxysterole über die Verabreichung eines moderat oxidierten Cholesterinpräparats physiologische Wirkungen entfalten, die einerseits das antioxidative Schutzsystem und andererseits den Lipidstoffwechsel unter besonderer Berücksichtigung der VLDL-Synthese und –Sekretion beeinflussen. Die Relevanz dieser Fragestellung ergab sich vor allem daraus, dass zwar die biologischen Wirkungen spezifischer Oxysterole vielfach untersucht wurden, jedoch die meisten Beobachtungen unter unphysiologischen Bedingungen (Verwendung isolierter, hochkonzentrierter Oxysterole *in vitro*, Verwendung massiv oxidierten Cholesterins *in vivo*) gemacht wurden. Zur Untersuchung der Fragestellung des zweiten Abschnitts wurden zwei Studien mit jeweils 36 männlichen Ratten durchgeführt, in denen in jeweils 4 Versuchsgruppen die Faktoren Cholesterin (oxidiertes vs. reines Cholesterin 5 g/kg Diät) und Diätfett (Lachsöl vs. Kokosfett 100 g/kg Diät) variiert wurden. Die Untersuchungen des zweiten Abschnitts ergaben bezüglich der physiologischen Wirkung oxidierten Cholesterins folgende Ergebnisse:

*1       Wirkung auf das antioxidative Schutzsystem:* Die Vitamin E-Konzentration in der Leber wurde nicht durch die Art des Cholesterins, jedoch durch die Art des Fettes beeinflusst. Ratten, die Kokosfett als Diätfett erhielten, wiesen höhere Konzentrationen an Vitamin E auf als Ratten, die Lachsöl als Diätfett erhielten. Die Verabreichung von oxidiertem Cholesterin führte im Vergleich zu reinem Cholesterin zu einer erhöhten Aktivität der Glutathionperoxidase und zu verminderten Konzentrationen an Glutathion (gesamt und reduziert), hatte jedoch keinen Einfluss auf die Aktivität der Glukose-6-phosphat-Dehydrogenase. Die mRNA-Konzentrationen der Glutathionperoxidase und der Superoxiddismutase wurden durch die Fütterung oxidierten Cholesterins gegenüber reinem Cholesterin erhöht. Lediglich tendenziell erhöht wurde die mRNA-Konzentrationen der Glutathionreduktase durch Verabreichung oxidierten im Vergleich zu reinen Cholesterins. Bezüglich der Aktivitäten und mRNA-Konzentrationen antioxidativer Enzyme sowie der Konzentrationen an Glutathion (gesamt und reduziert) wurden keine Interaktionen zwischen der Art des Cholesterins und der Art des Diätfettes ermittelt.

*2       Wirkung auf den Lipidstoffwechsel und auf Parameter der VLDL-Synthese und – Sekretion:* Die Fütterung oxidierten Cholesterin führte im Vergleich zu reinem Cholesterin zu verminderten Lipidkonzentrationen in Plasma und der VLDL-Fraktion. Sowohl die Parameter der hepatischen Lipogenese (Genexpression des *sterol regulatory element binding proteins*-1c

und Aktivität der Glukose-6-phosphat-Dehydrogenase) als auch die hepatischen Lipidkonzentrationen wurden durch Fütterung oxidierten im Vergleich zu reinem Cholesterin nicht beeinflusst. Die Anteile der Arachidonsäure in den hepatischen Lipidfraktionen waren bei den Ratten, deren Diät oxidiertes Cholesterin enthielt, gegenüber den Ratten, deren Diät reines Cholesterin enthielt, erhöht. Die Genexpression des Apolipoprotein B wurde durch die Art des Cholesterins nicht beeinflusst, während die Genexpression des Mikrosomalen Triglyzerid-Transferproteins, das essentiell für die VLDL-Synthese und –Sekretion ist, durch oxidiertes Cholesterin im Vergleich zu reinem Cholesterin vermindert wurde.

Als Fazit des zweiten Untersuchungsabschnitts ergab sich, dass die Verabreichung oxidierten Cholesterins einerseits oxidativen Stress in der Leber von Ratten erzeugt und andererseits den Fettsäuremetabolismus beeinflusst und die Lipidkonzentrationen in Plasma und VLDL vermindert, was möglicherweise auf eine verminderte Transkription des MTP-Gens zurückzuführen ist.

Insgesamt zeigen die Ergebnisse der vorliegenden Arbeit, dass die Konzentrationen an pathophysiologisch bedeutsamen Oxysterolen im Organismus durch die Diätzusammensetzung ungünstig beeinflusst werden können. Insbesondere eine Erhöhung der 7β-OH-Chol-Konzentrationen im Organismus durch Gabe von Fischöl in Verbindung mit einer geringen Vitamin E-Zufuhr oder einer hohen Eisenzufuhr ist kritisch zu bewerten, da erhöhte Plasmakonzentrationen an 7β-OH-Chol offensichtlich nicht nur ein erhöhtes Arterioskleroserisiko, sondern auch mit dem Lungenkrebsrisiko positiv assoziiert ist. Die festgestellten physiologischen Wirkungen oxidierten Cholesterins – insbesondere die Induzierung von oxidativem Stress, aber auch die Beeinflussung der Membraneigenschaften durch Inkorporierung der Oxysterole in die Biomembranen - stehen möglicherweise in Zusammenhang mit den vielfach beschriebenen pathophysiologischen Effekten von Oxysterolen bzw. oxidierten Cholesterins.

## 5.       **Summary**

The concentrations of nonenzymatically formed oxysterols such as 7β-hydroxycholesterol (7β-OH-chol) and 7-ketocholesterol (7-keto-chol) were shown to be suitable markers of *in vivo*-lipidperoxidation and/or oxidative stress. Oxidative stress was also shown to be induced by the diet composition in the mammalian organism. Therefore, the aim of the first part of the present work was to investigate the effects of different diet components and/or nutrients (fish oil, iron, vitamin E, oxidized cholesterol) on the concentrations of nonenzymatically formed oxysterols and 7β-OH-chol, respectively. Rats were used as modell objects as they are susceptible to the induction of oxidative stress and/or are suitable to study the *in vivo*-formation of oxysterols. Thus, three studies with male rats were carried out in the first part of the present work. In the first study, the experimental factors fat (salmon oil vs. lard 100g/kg diet) and iron (50 vs. 500 mg iron-supplement/kg diet) were varied. The second study was performed in two separated experiments in parallel; in the first experiment the experimental factors fat (salmon oil vs. coconut oil 100g/kg diet) and vitamin E (40 vs. 240 mg α-tocopherol equivalents (α-TE/kg diet) and in the second experiment the factors cholesterol (oxidized vs. pure cholesterol 5g/kg diet), fat (salmon oil vs. coconut oil 100g/kg diet) and vitamin E (40 vs. 240 mg α-TE/kg diet) were varied. In the third study the factors fat (salmon oil vs. coconut oil 100g/kg diet) and vitamin E (10, 20, 40 vs. 240 mg α-TE/kg diet) were varied. The following results were obtained from the studies of the first part of the present work:

*1       Effect of fish oil:* In the first study, the hepatic concentrations of all oxysterols measured were one- to threefold higher in the rats fed salmon oil than in the rats fed lard. In the second study, the concentrations of 7β-OH-chol in liver, plasma, LDL, and erythrocytes were also higher in the rats fed salmon oil than in the rats fed coconut oil, irrespective of the dietary vitamin E concentration. In the third study, only the concentrations of 7β-OH-chol in the liver of rats, whose diets contained salmon oil as dietary fat, were higher than in the liver of rats, whose diets contained coconut oil as dietary fat, irrespective of the dietary vitamin E concentration. In opposite, the effect of the salmon oil on the concentrations of 7β-OH-chol in plasma, LDL, and erythrocytes, in the third study, was dependent on the dietary vitamin E. Only the rats fed salmon oil diets with 10 and 20 mg α-TE/kg diet had higher concentrations of 7β-OH-chol compared with the corresponding rats fed coconut oil.

*2       Effect of iron:* The effect of dietary iron on the oxysterol concentrations investigated was dependent on the dietary fat. Rats fed salmon oil and the high iron diet had higher hepatic

concentrations of 7β-OH-chol, 7-keto-chol, and cholestanetriol than rats fed salmon oil and the low iron diet. In contrast, in the rats, whose diets contained lard as dietary fat, those oxysterol concentrations were not influenced by the iron concentration of the diet. The concentrations of the epimeric cholesterol epoxides were not influenced by the iron concentration of the diet.

*3*       *Effect of vitamin E:* In the second study, only the concentrations of 7β-OH-chol in plasma, but not in the liver, LDL, and erythrocytes were influenced by the dietary vitamin E. An increase in the dietary vitamin E concentration from 40 to 240 mg α-TE/kg diet reduced the concentration of 7β-OH-chol in plasma of the rats fed both types of fat, either coconut oil or salmon oil. In the third study, however, the concentrations of 7β-OH-chol were not affected by the dietary vitamin E in the rats fed coconut oil, but in the rats fed salmon oil. The rats fed salmon oil and the lowest dietary vitamin E concentration (10 mg α-TE/kg diet) had the highest concentrations of 7β-OH-chol in all samples analyzed. Increasing the dietary vitamin E concentration in the diet continuously from 10 to 40 mg α-TE/kg reduced the concentrations of 7β-OH-chol in liver, plasma, LDL, and erythrocytes. Increasing the dietary vitamin E from 40 to 240 mg α-TE/kg diet did not further decrease the concentrations of 7β-OH-chol.

*4*       Effect of oxidized cholesterol: In the third study, feeding diets containing oxidized cholesterol increased the concentrations of 7β-OH-Chol in all samples analyzed of the rats fed coconut oil diets, but in the rats fed salmon oil diets only in liver and plasma. An additional increase in the dietary vitamin E concentration reduced the concentration of 7β-OH-chol only in plasma.

In conclusion, the results of the first part of the present work showed that feeding dietary fats rich in n-3-PUFA, especially in combination with a low vitamin E supply, leads to higher concentrations of nonenzymatically formed oxysterols and 7β-OH-Chol, respectively, as compared with feeding dietary fats low in n-3-PUFA. A high iron diet increases the concentration of 7β-OH-Chol and other nonenzymatically formed oxysterols only in combination with a fat rich in n-3-PUFA. The effect of oxidized cholesterol on the concentration of 7β-OH-Chol is independent on the type of dietary fat.

The aim of the second part of the present work was to determine the effects of exogenous oxysterols, as supplied by feeding a moderately oxidized cholesterol preparation, on specific parameters of the antioxidative defense system and on lipid metabolism with specific consideration of parameters of VLDL assembly and secretion. This aim arose from the fact, that the biological effects of specific oxysterols were in fact studied in many cases, but mostly under non physiologic conditions (use of isolated, high concentrated oxysterols *in vitro*; use

of extremely oxidized cholesterol *in vivo*). Therefore, in the second part of the present work, two studies each with 36 male rats were carried out varying the experimental factors cholesterol (oxidized vs. pure cholesterol 5g/kg diet) and dietary fat (salmon oil vs. coconut oil 100g/kg diet) in 4 treatment groups. The following results were obtained from the studies of the second part of the present work:

*1*        *Effect on the antioxidative defense system:* The hepatic cocentrations of vitamin E were not affected by the type of cholesterol, but by the type of dietary fat. Rats fed the coconut oil diets had higher concentrations of vitamin E than rats fed the salmon oil diets. Feeding diets containing oxidized cholesterol as compared with feeding diets containing pure cholesterol increased the activity of glutathione peroxidase and reduced the concentrations of glutathione (total and reduced), but did not influence the activity of glucose-6-phosphate dehydrogenase. Feeding oxidized cholesterol compared with feeding pure cholesterol increased mRNA concentrations of both, glutathione peroxidase and superoxide dismutase; in addition, glutathione reductase was only slightly increased by feeding oxidized cholesterol compared with pure cholesterol. No interactions were found between the dietary cholesterol and the dietary fat regarding the activities and mRNA concentrations of antioxidative enzymes and the concentrations of glutathione (total and reduced).

*2*        *Effect on lipid metabolism and on parameters of VLDL assembly and secretion:* Feeding oxidized cholesterol reduced concentrations of lipids in plasma and VLDL compared with feeding pure cholesterol. Both, the parameters of hepatic lipogenesis (gene expression of sterol regulatory protein-1c and activity of glucose-6-phosphate dehydrogenase) and the hepatic lipid concentrations were not influenced by the type of dietary cholesterol. The proportions of arachidonic acid were higher in the rats fed oxidized cholesterol than in the rats fed pure cholesterol. Gene expression of apolipoprotein B was not affected by the type of cholesterol, whereas oxidized cholesterol reduced the gene expression of microsomal triglycerid transfer protein, which is essential for the assembly and secretion of VLDL, compared with pure cholesterol.

In conclusion, the results from the second part of the present work showed that dietary oxidized cholesterol induced oxidative stress in the liver and influenced fatty acid metabolism and reduced the concentrations of lipids in plasma and VLDL, which is possibly attributed to a reduced transcription of the MTP gene.

To summarize, the results of the present work showed that the concentrations of pathophysiologic relevant oxysterols in the organism may be unfavourably influenced by the diet composition. In particular, an increase in the concentration of 7β-OH-Chol by feeding

fish oil in combination with a low vitamin E diet or a high iron diet must be considered critically concerning the observation that high plasma concentrations of 7β-OH-Chol are not only associated with a higher risk of atherosclerosis, but also with a higher risk of lung cancer. Therefore, the observed physiologic effects of oxidized cholesterol – in particular the induction of oxidative stress, but also the altered membrane fluidity caused by the incorporation of oxysterols into the membranes – are possibly related to the multiple reported pathophysiologic effects of oxysterols and oxidized cholesterol, respectively.

## 6. Literaturverzeichnis

ADACHI, J., KUDO, R., UENO, Y., HUNTER, R., RAJENDRAM, R., WANT, E., PREEDY, V.R. (2001) Heart 7-hydroperoxycholesterol and oxysterols are elevated in chronically ethanol-fed rats. J Nutr 131: 2916-2920.

ALEYNIK, S.I., LEO, A.M., ALEYNIK, M.K., LIEBER, C.S. (1999) Alcohol induced pancreatic oxidative stress: protection by phospholipid repletion. Free Radic Biol Med 26: 609-619.

ARIYOSHI, K., ADACHI, J., ASANO, M., UENO, Y., RAJENDRAM, R., PREEDY, V.R. (2002) Effect of chronic ethanol feeding on oxysterols in rat liver. Free Radic Res 36: 661-666.

ATALAY, M., LAAKSONEN, D.E., KHANNA, S., KALISTE-KORHONEN, E., HÄNNINEN, O., SEN, C.K. (2000) Vitamin E regulates changes in tissue antioxidants induced by fish oil and acute exercise. Med Sci Sports Exerc 32: 601-607.

AUST, S.D., MOREHOUSE, L.A., THOMAS, C.E. (1986) Role of metals in oxygen radical reactions. Free Radic Biol Med 1: 3-25.

AVIRAM, M. (2000) Review on human studies on oxidative damage and antioxidant protection related to cardiovascular diseases. Free Radic Res 33 Suppl: S85-97.

BACON, B.R., TAVILL, A.S., BRITTENHAM, G.M., PARK, C.H., RECKNAGEL, R.O. (1983a) Hepatic lipid peroxidation in vivo in rats with chronic iron overload. J Clin Invest 71: 429-439.

BACON, B.R., BRITTENHAM, G.M., TAVILL, A.S., MCLAREN, C.E., PARK, C.H., RECKNAGEL, R.O. (1983b) Hepatic lipid peroxidation in vivo in rats with chronic dietary iron overload is dependent on hepatic iron concentration. Trans Assoc Am Physicians 96: 146-154.

BANSAL, G., SINGH, U., BANSAL, M.P. (2001) Effect of 7β-hydroxycholesterol on cellular redox status and heat shock protein 70 expression in macrophages. Cell Physiol Biochem 11: 241-246.

BENNETT, A.J., BILLETT, M.A., SALTER, A.M., WHITE, D.A. (1995) Regulation of hamster hepatic microsomal triglyceride transfer protein mRNA levels by dietary fats. Biochem Biophys Res Commun 212: 473-478.

BENNETT, A.J., BRUCE, J.S., SALTER, A.M., WHITE, D.A., BILLETT, M.A. (1996) Hepatic microsomal triglyceride transfer protein messenger RNA concentrations are increased by dietary cholesterol in hamsters. FEBS Lett 394: 247-250.

BERGSETH, S., CHRISTIANSEN, E.N., BREMER, J. (1986) The effect of feeding fish oils, vegetable oils and clofibrate on the ketogenesis from long-chain fatty acids in hepatocytes. Lipids 21: 508-514.

BERLINER, J.A., HEINECKE, J.W. (1996) The role of oxidized lipoproteins in atherogenesis. Free Radic Biol Med 20: 707-727.

BIESALSKI, H.K., FRANK, J. (1995) Antioxidanzien in der Ernährung und ihre Bedeutung für die anti-/prooxidative Balance im Immunsystem. Immun Infekt 23: 166-173.

BJÖRKHEM, I. (2002) Do oxysterols control cholesterol homeostasis? J Clin Invest 110: 725-730.

BJÖRKHEM, I., DICZFALUSY, U. (2002) Oxysterols: friends, foes, or just fellow passengers? Arterioscler Thromb Vasc Biol 22: 734-742.

BJÖRKHEM, I., EINARSSON, K., JOHANSSON, G. (1968) Formation and metabolism of 3β-hydroxycholest-5-en-7-one and cholest-5-ene-3β,7β-diol. Acta Chem Scand 22: 1595-1605.

BJÖRKHEM, I., HENRIKSSON-FREYSCHUSS, A., BREUER, O., DICZFALUSY, U., BERGLUND, L., HENRIKSSON, P. (1991) The antioxidant butylated hydroxytoluene protects against atherosclerosis. Arterioscler Thromb 11: 15-22.

BJÖRKHEM, I., LÜTJOHANN, D., BREUER, O., SAKINIS, A., WENNMALM, A. (1997) Importance of a novel oxidative mechanism for elimination of brain cholesterol. Turnover of cholesterol and 24(S)-Hydroxycholesterol in rat brain as measured with $^{18}O_2$ techniques in vivo and in vitro. J Biol Chem 48: 30178-30184.

BJÖRKHEM, I., MEANEY, S., DICZFALUSY, U. (2002) Oxysterols in human circulation: which role do they have? Curr Opin Lipidol 13: 247-253.

BLACHE, D., RODRIGUEZ, C., DAVIGNON, J. (1995) Pro-oxidant effects of 7-hydroperoxycholest-5-en-3 beta-ol on the copper-initiated oxidation of low density lipoprotein. FEBS Lett. 357:135-139.

BOISSONNEAULT, G.A., HEININGER, H.J., (1985) 25-Hydroxycholesterol induced elevations in $^{45}Ca$ uptake: permeability changes in P815 cells. J Cell Physiol 125: 471-475.

BOISSONNEAULT, G.A., HENNIG, B., OUYANG, C.M. (1991a) Oxysterols, cholesterol biosynthesis, and vascular endothelial cell monolayer barrier function. Proc Soc Exp Biol Med 196: 338-343.

BOISSONNEAULT, G.A., HENNIG, B., WANG, Y., OUYANG, C.M., KRAHULIK, K., CUNNUP, L., OELTGEN, P.R. (1991b) Effect of oxysterol-enriched low-density

lipoprotein on endothelial barrier function in culture: low-density lipoproteins. Ann Nutr Metab 35: 226-232.

BÖSTROM, H. (1983) Binding of cholesterol to cytochromes P-450 from rabbit liver microsomes. J Biol Chem 258: 15091-15094.

BRANDSCH, C., RINGSEIS, R., EDER, K. (2002) High dietary iron concentrations enhance the formation of cholesterol oxidation products in the liver of adult rats fed salmon oil with minimal effects on antioxidant status. J Nutr 132: 2263-2269.

BRENNER, R.R. (1981) Nutritional and hormonal factors influencing desaturation of essential fatty acids. Prog Lipid Res 20: 41-47.

BRENNER, R.R. (1990) Role of cholesterol in the microsomal membrane. Lipids 25: 581-585.

BREUER, O., DZELETOVIC, S., LUND, E., DICZFALUSY, U. (1996) The oxysterols cholest-5-ene-3β,4α-diol, cholest-5-ene-3β,4β-diol and cholestane-3β,5α,6α-triol are formed during in vitro oxidation of low density, and are present in human atherosclerotic plaques. Biochim Biophys Acta 1302: 145-152.

BREUER, O., SUDJANA-SUGIAMAN, E., EGGERTSEN, G., CHIANG, J.Y.L., BJÖRKHEM, I. (1993) Cholesterol 7α-hydroxylase is up-regulated by the competitive inhibitor 7-oxocholesterol in rat liver. Eur J Biochem 215: 705-710.

BRIGELIUS-FLOHÉ, R., TRABER, M.G. (1999) Vitamin E: function and metabolism. FASEB J 13: 1145-1155.

BRIGGS, M.R., YOKOYAMA, C., WANG, X., BROWN, M.S., GOLDSTEIN, J.L. (1993) Nuclear protein that binds sterol regulatory element of low density lipoprotein receptor promoter. I. Identification of the protein and delineation of its target nucleotide sequence. J Biol Chem 268: 14490-14496.

BRITTON, R.S., BACON, B.R., RECKNAGEL, R.O. (1987) Lipid peroxidation and associated hepatic organelle dysfunction in iron overload. Chem Phys Lipids 45: 207-239.

BROWN, A.J., DEAN, R.T., JESSUP, W. (1996) Free and esterified oxysterols: formation during copper-oxidation of low density lipoprotein and uptake by macrophages. J Lipid Res 37: 320-335.

BROWN, A.J., LEONG, S.L., DEAN, R.T., JESSUP, W. (1997) 7-Hydroperoxycholesterol and its products in oxidized low density lipoprotein and human atherosclerotic plaque. J Lipid Res 38: 1730-1745.

BRUNET, S., THIBAULT, L., DELVIN, E., YOTOV, W., BENDAYAN, M., LEVY, E. (1999) Dietary iron overload and induced lipid peroxidation are associated with impaired plasma lipid transport and hepatic sterol metabolism in rats. Hepatology 29: 1809-1817.

BUCKINGHAM, K.W. (1985) Effect of dietary polyunsaturated/saturated fatty acid ratio and dietary vitamin E on lipid peroxidation in the rat. J Nutr 115: 1425-1435.

BUDDECKE, E. (1994) Grundriß der Biochemie. 9. Aufl. Berlin, New York: de Gruyter Verlag.

BULUR, H., GOKKUSA, C., UYSAL, M. (1986) Erythrocyte lipid peroxidation and anemia in cholesterol-fed rabbits. Nutr Rep Int 33: 247-251.

BURR, M.L., FEHILY, A.M., GILBERT, J.F., ROGERS, S., HOLLIDAY, R.M., SWEETNAM, P.M., ELWOOD, P.C., DEADMAN, N.M. (1989) Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). Lancet 2(8666): 757-61.

BURTON, G.W., INGOLD, K.U. (1986) Vitamin E: application of the principles of physical organic chemistry to the exploration of structure and function. Acc Chem Res 19: 194-201.

BURTON, G.W., TRABER, M.G. (1990) Vitamin E: antioxidant activity, biokinetics, and bioavailability. Annu Rev Nutr 10: 357-382.

BUTTRISS, J.L., DIPLOCK, A.T. (1988) The relationship between α-tocopherol and phospholipid fatty acids in rat liver subcellular membrane fractions. Bioch Biophys Acta 962: 81-90.

CANTWELL, H., DEVERY, R. (1998) The response of the antioxidant defense system in rat hepatocytes challenged with oxysterols is modified by Covi-ox. Cell Biol Toxicol 14: 401-409.

CARPENTER, K.L.H., BALLANTINE, J.A., FUSSELL, B., ENRIGHT, J.H., MITCHINSON, M.J. (1990) Oxidation of cholesteryl linoleate by human monocyte-macrophages in vitro. Atherosclerosis 83: 217-229.

CARPENTER, K.L.H., TAYLOR, S.E., VAN DER VEEN, C., WILLIAMSON, B.K., BALLANTINE, J.A., MITCHINSON, M.J. (1995) Lipids and oxidised lipids in human atherosclerotic lesions at different stages of development. Biochim Biophys Acta 1256: 141-150.

CARPENTER, K.L.H., TAYLOR, S.E., BALLANTINE, J.A., FUSSELL, B., HALLIWELL, B., MITCHINSON, M.J. (1993) Lipids and oxidised lipids in human atheroma and normal aorta. Biochim Biophys Acta 1167: 121-130.

CARPENTER, K.L.H., WILKINS, G.M., FUSSELL, B., BALLANTINE, J.A., TAYLOR, S.E., MITCHINSON, M.J., LEAKE, D.S. (1994) Production of oxidized lipids during modification of low-density lipoprotein by macrophages or copper. Biochem J 304: 625-633.

CARUSO, D., RASETTI, M.F., DE ANGELIS, L., GALLI, G. (1996) Identification of 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide in human oxidized LDL. Chem Phys Lipids 79: 181-186.

CATHCART, M.K., MOREL, D.W., CHISOLM, G.M. (1985) Monocytes and neutrophils oxidize low density lipoprotein making it cytotoxic. J Leukocyte Biol 38: 341-350.

CHANG, Y.H., ABALLA, D.S.P., SEVANIAN, A. (1997) Characterization of cholesterol oxidation products formed by oxidative modification of low-density lipoprotein. Free Radic Biol Med 23: 202-214.

CHAO, P.M., CHAO, C.Y., LIN, F.J., HUANG, C. (2001) Oxidized frying oil up-regulates hepatic acyl-CoA oxidase and cytochrome P450-4A1 genes in rats and activates PPARalpha. J Nutr 131: 3166-3174.

CHAPMAN, R.W., MORGAN, M.Y., LAULICHT, M., HOFFBRAND A.V., SHERLOCK, S. (1982) Hepatic iron stores and markers of iron overload in alcoholics and patients with idiopathic hemochromatosis. Dig Dis Sci 27: 909-916.

CHAUTAN, M., CHANUSSOT, F., PORTUGAL, H., PAULI, A.M., LAFONT, H. (1990) Effects of salmon oil and corn oil on plasma lipid level and hepato-biliary cholesterol metabolism in rats. Biochim Biophys Acta 1046: 40-45.

CHEN, L.-C., BOISSONNEAULT, G., HAYEK, M.G., CHOW, C.K. (1993) Dietary fat effects on hepatic lipid peroxidation and enzymes of H2O2 metabolism and NADPH generation. Lipids 28: 657-662.

CHISOLM, G.M., MA, G., IRWIN, K.C., MARTIN, L.L, GUNDERSON, K.G., LINBERG, L.F., MOREL, D.W., DICORLETO, P.E. (1994) 7β-Hydroperoxycholest-5-en-3β-ol, a component of human atherosclerotic lesions, is the primary cytotoxin of oxidized human low density lipoprotein. Proc Natl Acad Sci USA 91: 11452-11456.

CHO, S.H., CHOI, Y.S. (1994) Lipid peroxidation and antioxidant status is affected by different vitamin E levels when feeding fish oil. Lipids 29: 47-52.

CHO, B.H.S., EGWIM, P.O., FAHEY, G.C. (1986) Effects of pure and auto-oxidized forms of cholesterol on plasma, liver lipids and hepatic lipogenesis in chicks. Comp Biochem Physiol 83B: 767-770.

CHO, S.H., IM, J.G., CHOI, Y.S., SON, Y.S., CHUNG, M.H. (1995) Lipid peroxidation and 8-hydroxydeoxyguanosine formation in rats fed fish oil with different levels of vitamin E. J Nutr Sci Vitaminol 41: 61-72.

CHOW, C.K., IBRAHIM, W., WEI, Z., CHAN, A.C. (1999) Vitamin E regulates mitochondrial hydrogen peroxide generation. Free Radic Biol Med 27: 580-587.

COOK, J.D., LIPSCHITZ, D.A., MILES, L.E., FINCH, C.A. (1974) Serum ferritin as a measure of iron stores in normal subjects. Am J Clin Nutr 27: 681-687.

COOK, J.D., SKIKNE, B.S., BAYNES, R.D. (1994) Iron deficiency: the global perspective. Adv Exp Med Biol 356: 219-228.

CROSS, C.E., HALLIWELL, B., BORISH, E.T., PRYOR, W.A., SAUL, R.L., MCCORD, J.M., HARMAN, D. (1987) Oxygen radicals and human disease. Ann Intern Med 107: 526-545.

CUCHEL, M. ET AL. (1997) Lovastatin decreases de novo cholesterol synthesis and LDL apo B-100 production rates in combined-hyperlipidemic males. Arterioscler Thromb Vasc Biol 17: 1910-1917.

DABBAGH, A.J., MANNION, T., LYNCH, S.M., FREI, B. (1994) The effect of iron overload on rat plasma and liver oxidant status in vivo. Biochem J 300: 799-803.

D'AQUINO, M., BENEDETTI, P.C., DI FELICE, M., GENTILI, V., TOMASSI, G., MAIORINO, M., URSINI, F. (1991) Effect of fish oil and coconut oil on antioxidant defence system and lipid peroxidation in rat liver. Free Radic Res Commun 12-13 Pt 1:147-52.

DAVIES, M.J., SLATER, T.F. (1987) Studies on the metal-ion and lipoxygenase-catalysed breakdown of hydroperoxides using electron-spin-resonance spectroscopy. Biochem J 245: 167-173.

DE, A.K., DARAD, R. (1988) Physiological antioxidants and antioxidative enzymes in vitamin E-deficient rats. Toxicol Lett 44: 47-54.

DE LORGERIL, M., SALEN, P., MARTIN, J.L., MONJAUD, I., DELAYE, J., MAMELLE, N. (1999) Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart Study. Circulation 99: 779-785.

DGE, ÖGE, SGE, SVE (2000) Referenzwerte für die Nährstoffzufuhr. 1.Aufl. Umschau, Braus, Frankfurt (Main).

DILLARD, C.J., GAVINO, V.C., TAPPEL, A.L. (1983) Relative antioxidant effectiveness of α-tocopherol and γ-tocopherol in iron-loaded rats. J Nutr 113: 2266-2273.

DILLARD, C.J., TAPPEL, A.L. (1979) Volatile hydrocarbon and carbonyl products of lipid peroxidation: a comparison of pentane, ethane, hexanal, and acetone as in vivo indices. Lipids 14: 989-995.

DONOVAN, D.H., MENZEL, D.B. (1978) Mechanisms of lipid peroxidation: iron catalysed decomposition of fatty acid hydroperoxides as the basis of hydrocarbon evolution in vivo. Experientia 34: 775-776.

DRAPER, H.H., MC GIRR, L.G., HADLEY, M. (1986) The metabolism of malondialdehyde, Lipids 21: 305-307.

DUANE, P., RAJA, K.B., SIMPSON, R.J., PETERS, T.J. (1992) Intestinal iron absorption in chronic alcoholics. Alcohol Alcohol 27: 539-544.

DZELETOVIC, S., BABIKER, A., LUND, E., DICZFALUSY, U. (1995) Time course of oxysterol formation during in vitro oxidation of low density lipoprotein. Chem Phys Lipids 78: 119-128.

EDER, K., FLADER, D., HIRCHE, F., BRANDSCH, C. (2002) Excess dietary vitamin E lowers the activities of antioxidative enzymes in erythrocytes of rats fed salmon oil. J Nutr 132: 3400-3404.

EDWARDS, P.A., ERICSSON, J. (1998) Signaling molecules derived from the cholesterol biosynthetic pathway: mechanisms of action and possible roles in human disease. Curr Opin Lipidol 9: 433-440.

ELMADFA, I., LEITZMANN C. (1990) Vitamin E (Tocopherole). In: Elmadfa, Leitzmann (Hrsg.) Ernährung des Menschen, 2.Aufl., Eugen Ulmer Verlag Stuttgart: 260.

EMANUEL, H.A., HASSEL, C.A., ADDIS, P.B., BERGMANN, S.D., ZAVORAL, J.H. (1991) Plasma cholesterol oxidation products (oxysterols) in human subjects fed a meal rich in oxysterols. J Food Sci 56: 843-847.

ENOMOTO, A., ITOH, K., NAGAYOSHI, E., HARUTA, J., KIMURA, T., O'CONNOR, T., HARADA, T., YAMAMOTO, M. (2001) High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. Toxicol Sci 59: 169-177.

ERICKSON, S.A., COOPER, A.D., MATSUI, S.M., GOULD, R.G. (1977) 7-Ketocholesterol, its effects on hepatic cholesterogenesis and its hepatic metabolism in vivo and in vitro. J Biol Chem 252: 5186-5193.

ESTERBAUER, H. (1996) Estimation of peroxidative damage. Path Biol 44: 25-28.

ESTERBAUER, H., DIEBER-ROTHENEDER, M., STRIEGL, G., WAEG, G. (1991) Role of vitamin E in preventing the oxidation of low density lipoprotein. Am J Clin Nutr 53: 314S-321S.

FIELD, F.J., ALBRIGHT, E.J., MATHUR, S.N. (1987) Effect of dietary n-3 fatty acids on HMG-CoA reductase and ACAT activities in liver and intestine of the rabbit. J Lipid Res 28: 50-58.

FLADER, D., BRANDSCH, C., HIRCHE, F., EDER, K. Effects of megadoses of dietary vitamin E on the antioxidant status of rats fed lard or salmon oil. Int J Vitam Nutr Res (Manuskript angenommen zur Publikation).

FORD, C., WELLS, F.E., ROGERS, J.N. (1995) Assessment of iron status in association with excess alcohol consumption. Ann Clin Biochem 32: 527-531.

FRANKEL, E.N. (1991) Review: Recent advances in lipid oxidation. J Sci Agric 54: 495-511.

FREI, B., STOCKER, R., AMES, B.N. (1988) Antioxidant defenses and lipid peroxidation in human blood plasma. Proc Natl Acad Sci U S A 85: 9748-9752.

FUJITA, T., ADACHI, J., UENO, Y., PETERS, T.J., PREEDY, V.R. (2002) Chronic ethanol feeding increases 7-hydroperoxycholesterol and oxysterols in rat skeletal muscle. Metab 51: 737-742.

GARCIA-CRUSET, S., CARPENTER, K.L., GUARDIOLA, F., MITCHINSON, M.J. (1999) Oxysterols in cap and core of human advanced atherosclerotic lesions. Free Radic Res 30: 341-350.

GARCIA-CRUSET, S., CARPENTER, K.L., GUARDIOLA, F., STEIN, B.K., MITCHINSON, M.J. (2001) Oxysterol profiles of normal human arteries, fatty streaks and advanced lesions. Free Radic Res 35: 31-41.

GARDNER (1987) Reactions of hydroperoxides – products of high molecular weight. In: Autoxidation of unsaturated lipids. Chan, H.W.-S. (Ed.). Academic Press, London. 51-93.

GARG, M.L., SNOSWELL, A.M., SABINE, J.R. (1986) Influence of dietary cholesterol on desaturase enzymes of rat liver microsomes. Prog Lipid Res 25: 639-644.

GINSBERG, H.N., LE, N.A., SHORT, M.P., RAMAKRISHNAN, R., DESNICK, R.J. (1987) Suppression of apolipoprotein B production during treatment of cholesteryl ester storagee disease with lovastatin. Implications for regulation of apolipoprotein B synthesis J Clin Invest 80: 1692-1697.

GLEI, M., LATUNDE-DADA, G.O., KLINDER, A., BECKER, T.W., HERMANN, U., VOIGT, K., POOL-ZOBEL, B.L. (2002) Iron-overload induces oxidative DNA damage in the human colon carcinoma cell line HT29 clone 19A. Mutat Res 519: 151-161.

GREGG, R.E., WETTERAU, J.R. (1994) The molecular basis of abetalipoproteinemia. Curr Opin Lipidol 5: 81-86.

GRIESMACHER, A., KINDHAUSER, M., ANDERT, S.E., SCHREINER, W., TOMA, C., KNOEBL, P., PIETSCHMANN, P., PRAGER, R., SCHNACK, C., SCHERNTHANER, G. ET AL. (1995) Enhanced serum levels of thiobarbituric-acid-reactive substances in diabetes mellitus. Am J Med 98: 469-475.

GUARDIOLA, F., CODONY, R., ADDIS, P.B., RAFECAS, M., BOATELLA, J. (1996) Biological effects of oxysterols: current status. Food Chem Toxicol 34: 193-211.

GUTTERIDGE, J.M.C. (1995) Peroxidation and antioxidants as biomarkers of tissue damage. Clin Chem 41: 1819-1828.

HAGAN, D.L., KIENZLE, B., JAMIL, H., HARIHARAN, N. (1994) Transcriptional regulation of human and hamster microsomal triglyceride transfer protein genes. Cell type-specific expression and response to metabolic regulators. J Biol Chem 269: 28737-28744.

HAGVE, T.A., LIE, O., GRONN, M. (1993) The effect of dietary n-3 fatty acids on osmotic fragility and membrane fluidity of human erythrocytes. Scand J Clin Lab Invest Suppl 215: 75-84.

HALLIWELL, B., GUTTERIDGE, J.M.C. (1986) Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. Arch Biochem Biophys 246: 501-514.

HALLIWELL, B., GUTTERIDGE, J.M.C. (1989) Free radicals in Biology and Medicine. Clarendon Press, Oxford, 186-276.

HALLIWELL, B., GUTTERIDGE, J.M.C. (1990) Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol 186: 1-8.

HALLIWELL, B., GUTTERIDGE, J.M. (1992) Biologically relevant metal ion-dependent hydroxyl radical generation. An update. FEBS Lett 307: 108-112.

HARATS, D., DABACH, Y., HOLLANDER, G., BEN-NAIM, M., SCHWARTZ, R., BERRY, E.M., STEIN, O., STEIN, Y. (1991) Fish oil ingestion in smokers and nonsmokers enhances peroxidation of plasma lipoproteins. Atherosclerosis 90: 127-139.

HARRIS, W.S. (1989) Fish oils and plasma lipid and lipoprotein metabolism in humans: A critical review. J Lipid Res 30: 785-807.

HEBBEL, R.P., EATON, J.W., BALASINGAM, M., STEINBERG, M.H. (1982) Spontaneous oxygen radical generation by sickle erythrocytes. J Clin Invest 70: 1253-1259.

HESSLER, J.R., MOREL, D.W., LEWIS, L.J., CHISOLM, G.M. (1983) Lipoprotein oxidation and lipoprotein-induced cytotoxicity. Arteriosclerosis 3: 215-222.

HODIS, H.N., CHAUHAN, A., HASHIMOTO, S., CRAWFORD, D.W., SEVANIAN, A. (1992) Probucol reduces plasma and aortic wall oxysterol levels in cholesterol-fed rabbits independently of its plasma cholesterol-lowering effects. Atherosclerosis 96: 125-134.

HODIS, H.N., CRAWFORD, D.W., SEVANIAN, A. (1991) Cholesterol feeding increases plasma and aortic tissue cholesterol oxide levels in parallel: further evidence for the role of cholesterol oxidation in atherosclerosis. Atherosclerosis 89: 117-126.

HODIS, H.N., HASHIMOTO, S., MACK, W.J., SEVANIAN, A. (2000) Probucol reduces oxysterol formation in hypertensive rabbits. Hypertension 36: 436-441.

HODIS, H.N., KRAMSCH, D.M., AVOGARO, P., BITTOLO-BON, G., CAZZOLATO, G., HWANG, J., PETERSON, H., SEVANIAN, A. (1994) Biochemical and cytotoxic characteristics of an in vivo circulating oxidized LDL. J Lipid Res 35: 669-677.

HOESCHEN, R.J. (1997) Oxidative stress and cardiovascular disease. Can J Cardiol 13: 1021-1025.

HORTON, J.D., GOLDSTEIN, J.L., BROWN, M.S. (2002) SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest 109: 1125-1131.

HOUGLUM, K., FILIP, M., WITZTUM, J.L., CHOJKIER, M. (1990) Malondialdehyde and 4-hydroxynonenal protein adducts in plasma and liver of rats with iron overload. J Clin Invest 86: 1991-1998.

HU, M.-L., FRANKEL, E.N., LEIBOWITZ, B.E., TAPPEL, A.L. (1989) Effect of dietary lipids and vitamin E on in vitro lipid peroxidation in rat liver and kidney homogenates. J Nutr 119: 1574-1582.

HUGHES, H., MATHEWS, B., LENZ, M.L., GUYTON, J.R. (1994) Cytotoxicity of oxidized LDL to porcine aortic smooth muscle cells is associated with the oxysterols 7-Ketocholesterol and 7-Hydroxycholesterol. Arterioscler Thromb 14: 1177-1185.

HUNT, J.V., SMITH, C.C., WOLFF, S.P. (1990) Autooxidative glycosilation and possible involvement of peroxides and free radicals in LDL modification by glucose. Diabetes 39: 1420-1424.

IBRAHIM, W., LEE, U.S., YEH, C.C., SZABO, J., BRUCKNER, G., CHOW, C.K. (1997) Oxidative stress and antioxidant status in mouse liver: effects of dietary lipid, vitamin E and iron. J Nutr 127: 1401-1406.

ISHII, K., ADACHI, J., TOMITA, M., KUROSAKA, M., UENO, Y. (2002) Oxysterols as indices of oxidative stress in man after paraquat ingestion. Free Radic Res 36: 163-168.

IULIANO, L. (2001) The oxidant stress hypothesis of atherogenesis. Lipids 36 Suppl: S41-44.

IULIANO, L., MICHELETTA, F., MARANGHI, M., FRATI, G., DICZFALUSY, U., VIOLI, F. (2001) Bioavailability of vitamin E as function of food intake in healthy subjects. Arterioscler Thromb Vasc Biol 21: e34-e37.

IULIANO, L., MICHELETTA, F., NATOLI, S., GINANNI CORRADINI, S., IAPPELLI, M., ELISEI, W., GIOVANNELLI, L., VIOLI, F., DICZFALUSY, U. (2003) Measurement of oxysterols and alpha-tocopherol in plasma and tissue samples as indices of oxidant stress status. Anal Biochem 312: 217-223.

JAATINEN, P., SAUKKO, P., HERVONEN, A. (1993) Chronic ethanol exposure increases lipopigment accumulation in human heart. Alcohol Alcohol 28: 559-569.

JAMIL, H., DICKSON, J.K. JR., CHU, C.H., LAGO, M.W., RINEHART, J.K., BILLER, S.A., GREGG, R.E., WETTERAU, J.R. (1995) Microsomal triglyceride transfer protein. Specificity of lipid binding and transport. J Biol Chem 270: 6549-6554.

JANERO, D.R. (1990) Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic Biol Med 1990: 515-540.

JAVOUHEY-DONZEL, A., GUENOT, L., MAUPOIL, V., ROCHETTE, L., ROCQUELIN, G. (1993) Rat vitamin E status and heart lipid peroxidation: effect of dietary alpha-linolenic acid and marine n-3 fatty acids. Lipids 28: 651-655.

JOHNSON, K.A., MORROW, C.J., KNIGHT, G.D., SCALLEN, T.J. (1994) In vivo formation of 25-hydroxycholesterol from endogenous cholesterol after a single meal, dietary cholesterol challenge. J Lipid Res 35: 2241-2253.

KALTWASSER, J.P., WERNER, E. (1989) Diagnosis and clinical evaluation of iron overload. Baillieres Clin Haematol 2: 363-385.

KATO, I., DNISTRIAN, A.M., SCHWARTZ, M., TONIOLO, P., KOENIG, K., SHORE, R.E., ZELENIUCH-JACQUOTTE, A., AKHMEDKHANOV, A., RIBOLI, E. (2000) Risk of iron overload among middle-aged women. Int J Vitam Nutr Res 70: 119-125.

KINALSKI, M., SLEDZIEWSKI, A., TELEJKO, B., ZARZYCKI, W., KINALSKA, I. (1999) Antioxidant therapy and streptozoticin-induced diabetes in pregnant rats. Acta Diabetol 36: 113-117.

KIRCHGESSNER, M., STANGL, G.I., REICHLMAYR-LAIS, A.M., EDER, K. (1994) The effects of dietary oils on the fatty acid composition and osmotic fragility of rat erythrocytes. Z Ernährungswiss 33: 146-158.

KORYTOWSKI, W., GEIGER, P.G., GIROTTI, A.W. (1996) Enzymatic reducibility in relation to cytotoxicity for various cholesterol hydroperoxides. Biochemistry 35: 8670-8679.

KOSUGI, K., MOREL, D.W., DICORLETO, P.E., CHISOLM, G.M. (1987) Toxicity of oxidized low-density lipoprotein to cultured fibroblasts is selective for S phase of the cell cycle. J Cell Physiol 130: 311-320.

KUBOW, S., GOYETTE, N., KERMASHA, S., STEWART-PHILLIP, J., KOSKI, K.G. (1996) Vitamin E inhibits fish oil-induced hyperlipidemia and tissue lipid peroxidation in hamsters. Lipids 31: 839-847.

KUCUK, O., LIS, L.J., DEY, T., MATA, R., WESTERMAN, M.P., YACHNIN, S., SZOSTEK, R., TRACY, D., KAUFFMAN, J.W., GAGE, D.A., ET AL. (1992) The effects of cholesterol oxidation products in sickle and normal red blood cell membranes. Biochim Biophys Acta 1103: 296-302.

LEAKE, D.S., RANKIN, S.M. (1990) The oxidative modification of low-density lipoproteins by macrophages. Biochem J 270: 741-748.

LEIBOWITZ, B.E., HU, M.-L., TAPPEL, A.L. (1990) Lipid peroxidation in rat tissue slices: effect of dietary vitamin E, corn oil-lard and menhaden oil. Lipids 25: 125-129.

LEMAIRE, S., LIZARD, G., MONIER, S., MIGUET, C., GUELDRY, S., VOLOT, F., GAMBERT, P., NEEL, D. (1998) Different patterns of IL-1beta secretion, adhesion

molecule expression and apoptosis induction in human endothelial cells treated with 7alpha-, 7beta-hydroxycholesterol, or 7-ketocholesterol. FEBS Letters 440: 434-439.

LINSEISEN, J., WOLFRAM, G. (1998a) Absorption of cholesterol oxidation products from ordinary foodstuff in humans. Ann Nutr Metab 42: 221-230.

LINSEISEN, J., WOLFRAM, G. (1998b) Origin, metabolism, and adverse health effects of cholesterol oxidation products. Fett/Lipid 100: 211-218.

LINSEISEN, J., WOLFRAM, G., MILLER, A.B. (2002) Plasma 7beta-hydroxycholesterol as a possible predictor of lung cancer risk. Cancer Epidemiol Biomarkers Prev 11: 1630-1637.

LIZARD, G., GUELDRY, S., SORDET, O., MONIER, S., ATHIAS, A., MIGUET, C., BESSEDE, G., LEMAIRE, S., SOLARY, E., GAMBERT, P. (1998) Glutathione is implied in the control of 7-ketocholesterol-induced apoptosis, which is associated with radical oxygen species production. FASEB J. 12: 1651-1663.

LIZARD, G., LEMAIRE, S., MONIER, S., GUELDRY, S., NÉEL, D., GAMBERT, P. (1997) Induction of apoptosis and of interleukin-1β secretion by 7β-hydroxycholesterol and 7-ketocholesterol: partial inhibition by Bcl-2 overexpression. FEBS Lett 419: 276-280.

LIZARD, G., MIGUET, C., BESSEDE, G., MONIER, S., GUELDRY, S., NEEL, D., GAMBERT, P. (2000) Impairment with various antioxidants of the loss of mitochondrial transmembrane potential and of the cytosolic release of cytochrome c occuring during 7-ketocholesterol-induced apoptosis. Free Radic Biol Med 28: 743-753.

LIZARD, G., MOISANT, M., CORDELET, C., MONIER, S., GAMBERT, P., LAGROST, L. (1996) Induction of apoptosis in endothelial cells treated with cholesterol oxides. Am J Pathol 148: 1625-1638.

LIZARD, G., MONIER, S., CORDELET, C., GESQUIÉRE, L., DECKERT, V., GUELDRY, S., LAGROST, L., GAMBERT, P. (1999) Characterization and comparison of the mode of cell death, apoptosis versus necrosis, induced by 7β-hydroxycholesterol and 7-ketocholesterol in the cells of the vascular wall. Arterioscler Thromb Vasc Biol 19: 1190-1200.

LUND, E., DICZFALUSY, U., BJÖRKHEM, I. (1992) On the mechanism of oxidation of cholesterol at C-7 in a lipoxygenase system. J Biol Chem 267: 12462-12467.

LYONS, N.M., WOODS, J.A., O´BRIEN, N.M. (2001) α-Tocopherol, nut not γ-tocopherol inhibits 7β-hydroxycholesterol-induced apoptosis in human U937 cells. Free Rad Res 35: 329-339.

LYONS, T.J. (1991) Oxidized low density lipoproteins: a role in the pathogenesis of atherosclerosis in diabetes? Diabetic Med 8: 411-419.

MACKENZIE, G.G., MACKENZIE, J.B., MCCOLLUM, E.V. (1941) Uncomplicated vitamin E deficiency in the rabbit and its relation to the toxicity of cod liver oil. J Nutr Sci 21: 225.

MAGGI-CAPEYRON, M.-F., CASES, J., BADIA, E., CRISTOL, J.-P., ROUANET, J.-M., BESANCON, P., LEGER, C.L., DESCOMPS, B. (2002) A diet high in cholesterol and deficient in vitamin E induces lipid peroxidation but does not enhance antioxidant enzyme expression in rat liver. J Nutr Biochem 13: 296-301.

MAHFOUZ, M.M., KAWANO, H., KUMMEROW, F.A. (1997) Effect of cholesterol-rich diets with and without added vitamins E and C on the severity of atherosclerosis in rabbits. Am J Clin Nutr 66: 1240-1249.

MAHFOUZ, M.M., KUMMEROW, F.A. (2000) Cholesterol-rich diets have different effects on lipid peroxidation, cholesterol oxides, and antioxidant enzymes in rats and rabbits. J Nutr Biochem 11: 293-302.

MALAVASI, B., RASETTI, M.F., ROMA, P., FOGLIATTO, R., ALLEVI, P., CATAPANO, A.L., GALLI, G. (1992) Evidence for the presence of 7-hydroperoxycholest-5-en-3 beta-ol in oxidized human LDL. Chem Phys Lipids 62: 209-214.

MALIS, C.D., WEBER, P.C., LEAF, A., BONVENTRE, J.V. (1990) Incorporation of marine lipids into mitochondrial membranes increases susceptibility to damage by calcium and reactive oxygen species: evidence for enhanced activation of phospholipase A2 in mitochondria enriched with n-3 fatty acids. Proc Natl Acad Sci U S A 87: 8845-8849.

MANTLE, D., PREEDY, V.R. (1999) Free radicals as mediators of alcohol toxicity. Adverse Drug React Toxicol Rev 18: 235-252.

MARKLUND, S.L. (1984) Extracellular superoxide dismutase and other superoxide dismutase isoenzymes in tissues from nine mammalian species. Biochem J 222: 649-655.

MCCLUSKEY, S., HALL, M., STANTON, C., DEVERY, R. (1999) Alpha-tocopherol inhibits oxidative stress induced by cholestanetriol and 25-hydroxycholesterol in porcine ovarian granulosa cells. Mol Cell Biochem 194: 217-225.

MEANEY, S., HASSAN, M., SAKINIS, A., LÜTJOHANN, D., VON BERGMANN, K., WENNMALM, A., DICZFALUSY, U., BJÖRKHEM, I. (2001) Evidence that the major oxysterols in human circulation originate from distinct pools of cholesterol: a stable isotope study. J Lipid Res 42: 70-78.

MEHTA, A., LOPEZ, L.M., WARGOVICH, T. (1987) Eicosapentaenoic acid: Its relevance in atherosclerosis and coronary artery diseases. Am J Cardiol 59: 155-161.

MENDELSOHN, D., MENDELSOHN, L., STAPLE, E. (1965) The catabolism in vitro of cholesterol: formation of the 7-epimeric hydroxycholesterols from cholesterol in rat liver. Biochem Biophys Acta 97: 379.

MEYDANI, S.N., SHAPIRO, A.C., MEYDANI, M., MACAULEY, J.B., BLUMBERG, J.B. (1987) Effect of age and dietary fat (fish oil, corn and coconut oils) on tocopherol status of C57BL/6Nia mice. Lipids 22: 345-350.

MIGUET-ALFONSI, C., PRUNET, C., MONIER, S., BESSEDE, G., LEMAIRE-EWING, S., BERTHIER, A., MENETRIER, F., NEEL, D., GAMBERT, P., LIZARD, G. (2002) Analysis of oxidative processes and of myelin figures formation before and after loss of mitochondrial transmembrane potential during 7beta-hydroxycholesterol and 7-ketocholesterol-induced apoptosis: comparison of various pro-apoptotic chemicals. Biochem Pharmacol 64: 527-541.

MILMAN, N., BYG, K.E., OVESEN, L., KIRCHHOFF, M., JURGENSEN, K.S. (2002) Iron status in Danish men 1984-94: a cohort comparison of changes in iron stores and the prevalence of iron deficiency and iron overload. Eur J Haematol 68: 332-340.

MINOTTI, G. (1993) Sources and role of iron in lipid peroxidation. Chem Res Toxicol 6: 134-146.

MIYAJIMA, H., ADACHI, J., KOHNO, S., TAKAHASHI, Y., UENO, Y., NAITO, T. (2001) Increased oxysterols associated with iron accumulation in the brains and visceral organs of acaeruloplasminaemia patients. QJM 94: 417-422.

MIYAJIMA, H., FUJIMOTO, M., KOHNO, S., KANEKO, E., GITLIN, J.D. (1998) CSF abnormalities in patients with aceruloplasminemia. Neurology 51: 1188-1190.

MIYAJIMA, H., TAKAHASHI, Y., SERIZAWA, M., KANEKO, E., GITLIN, J.D. (1996) Increased plasma lipid peroxidation in patients with aceruloplasminemia. Free Rad Biol Med 20: 757-760.

MOL, M.J.T.M., DE RIJKE, Y.B., DEMACKER, P.N.M., STALENHOEF, A.F.H. (1997) Plasma levels of lipid and cholesterol oxidation products and cytokines in diabetes

mellitus and cigarette smoking: effects of vitamin E treatment. Atherosclerosis 129: 169-176.

MOREL, D.W., HESSLER, J.R., CHISOLM, G.M. (1983) Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. J Lipid Res 24: 1070-1076.

MOREL, D.W., LIN, C.Y. (1996) Cellular biochemistry of oxysterols derived from the diet or oxidation in vivo. J Nutr Biochem 7: 495-506.

MORI, T.A., CROFT, K.D., PUDDEY, I.B., BEILIN, L.J. (1996) Analysis of native and oxidized low-density lipoprotein oxysterols using gas-chromatography-mass spectrometry with selective ion monitoring. Redox Report 21: 25-34.

MOURI, K., IKESU, H., ESAKA, T., IGARASHI, O. (1984) The influence of marine oil intake upon levels of lipids, α-tocopherol and lipid peroxidation in serum and liver of rats. J Nutr Sci Vitaminol 30: 307-318.

MUGGLI, R. (1994) Polyunsaturated fatty acids and the requirement of vitamin E. Fat Sci Technol 96: 17-19.

MUTO, T., TANAKA, J., MIURA, T., RIMURA, M. (1983) Chem Pharm Bull 31: 1561-1566.

NALBONE, G., LEONARDI, J., TERMINE, E., PORTUGAL, H., LECHENE, P., PAULI, A.M., LAFONT, H. (1989) Effects of fish oil, corn oil and lard diets on lipid peroxidation status and glutathione peroxidase activities in rat heart. Lipids 24: 179-186.

NESTEL, P.J. (1990) Effects of N-3 fatty acids on lipid metabolism. Annu Rev Nutr 10:149-167.

NICHOLS, G.M., BACON, B.R. (1989) Hereditary hemochromatosis: pathogenesis and clinical features of a common disease. Am J Gastroenterol. 84: 851-862.

NILSSON, A., HJELTE, L., STRANDVIK, B. (1992) Incorporation of dietary [$^{14}$C]arachidonic acid and [$^{3}$H]eicosapentaenoic acid into tissue lipids during absorption of a fish oil emulsion. J Lipid Res 33: 1295-1305.

NOSHIRO, M., NISHIMOTO, M., OKUDA, K. (1990) Rat liver cholesterol 7 alpha-hydroxylase. Pretranslational regulation for circadian rhythm. J Biol Chem 265: 10036-10041.

O´BRIEN, P.J. (1988) The fate and reactivity of lipid peroxides in: Chan, C.K. (Ed.): Cellular antioxidant defense mechanisms. Vol. 1, CRC Press, Inc., Boca Raton, 73-87.

O´CALLAGHAN, Y.C., WOODS, J.A., O´BRIEN, N.M. (2002) Characteristics of 7β-hydroxycholesterol-induced cell death in a human monocytic blood cell line, U937, and a human hepatoma cell line, HepG2. Toxicol in Vitro 16: 245-251.

O´CALLAGHAN, Y.C., WOODS, J.A., O´BRIEN, N.M. (1999) Oxysterol-induced cell death in U937 and HepG2 cells at reduced and normal serum concentrations. Eur J Nutr 38: 255-262.

O´DELL, B.L., BROWNING, J.D., REEVES, P.G. (1987) Zinc deficiency increases the osmotic fragility of rat erythrocytes. J Nutr 117: 1883-1889.

OSADA, K., INOUE, T., NAKAMURA, S., SUGANO, M. (1999) Dietary soybean protein moderates the deleterious disturbance of lipid metabolism caused by exogenous oxidized cholesterol in rats. Biochim Biophys Acta 1427: 337-350.

OSADA, K., KODAMA, T., CUI, L., ITO, Y., SUGANO, M. (1994b) Effects of dietary oxidized cholesterol on lipid metabolism in differently aged rats. Biosci Biotech Biochem 58: 1062-1069.

OSADA, K., KODAMA, T., MINEHIRA, K., YAMADA, K., SUGANO, M. (1996) Dietary protein modifies oxidized cholesterol-induced alterations of linoleic acid and cholesterol metabolism in rats. J Nutr 126: 1635-1643.

OSADA, K., KODAMA, T., NODA, S., YAMADA, K., SUGANO, M. (1995) Oxidized cholesterol modulates age-related change in lipid metabolism in rats. Lipids 30: 405-413.

OSADA, K., KODAMA, T., YAMADA, K., NAKAMURA, S., SUGANO, M. (1998) Dietary oxidized cholesterol modulates cholesterol metabolism and linoleic acid desaturation in rats fed high-cholesterol diets. Lipids 33: 757-764.

OSADA, K., SASAKI, E., SUGANO, M. (1994a) Lymphatic absorption of oxidized cholesterol in rats. Lipids 29: 555-559.

OTTO, D.A., BALTZELL, J.K., WOTTEN, J.T. (1992) Reduction in triacylglycerol by fish oil correlates with free fatty acid levels in ad libitum fed rats. Lipids 27: 1013-1017.

OTTO, D.A., TSAI, C.E., BALTZELL, J.K., WOOTEN, J.T. (1991) Apparent inhibition of hepatic triacylglycerol secretion, independent of synthesis, in high-fat fish oil-fed rats: role for insulin. Biochim Biophys Acta 1082: 37-48.

PALOZZA, P., SGARLATA, E., LUBERTO, C., PICCIONI, E., ANTI, M., MARRA, G., ARMELAO, F., FRANCESCHELLI, P., BARTOLI, G.M. (1996) n-3 fatty acids induce oxidative modifications in human erythrocytes depending on dose and duration of dietary supplementation. Am J Clin Nutr 64: 297-304.

PANIANGVAIT, P., KING, A.J., JONES, A.D., GERMAN, B.G. (1995) Cholesterol oxides in foods of animal origin. J Food Sci 60: 1159-1174.

PATEL, R.P., DICZFALUSY, U., DZELETOVIC, S., WILSON, M.T., DARLEY-USMAR, V.M. (1996) Formation of oxysterols during oxidation of low density lipoprotein by peroxynitrite, myoglobin, and copper. J Lipid Res 37: 2361-2371.

PEET, D.J., JANOWSKI, B.A., MANGELSDORF, D.J. (1998) The LXRs: a new class of oxysterol receptors. Curr Opin Genet Dev 8: 571-575.

PENG, S.K., HILL, J.C., MORIN, R.J., TAYLOR, C.B. (1985) Influence of cholesterol oxidation derivatives on membrane-bound enzymes in cultured aortic smooth muscle cells. Proc Soc Exp Biol Med 180: 126-132.

PENG, S.K., HU, B., MORIN, R.J. (1991) Angiotoxicity and atherogenicity of cholesterol oxides. J Clin Lab Anal 5: 144-152.

PENG, S.K., HU, B., CHEN, H., MORIN, R.J. (1992) Effects of cholesterol oxides on LDL receptors in cultured rabbit aortic smooth muscle cells. Cardiovasc Pathol 1: 141-145.

PENG S.K., TAYLOR, C.B., HILL, J.C., MORIN, R.J. (1985) Cholesterol oxidation derivatives and arterial endothelial damage. Atherosclerosis 54: 121-133.

PETTERSEN, K.S., BOBERG, K.M., STABURSVIK, A., PRYDZ, H. (1991) Toxicity of oxygenated cholesterol derivatives toward cultured human umbilical vein endothelial cells. Arterioscler Thromb 11: 423-428.

PORKKALA-SARATAHO, E., SALONEN, J.T., NYYSSONEN, K., KAIKKONEN, J., SALONEN, R., RISTONMAA, U., DICZFALUSY, U., BRIGELIUS-FLOHE, R., LOFT, S., POULSEN, H.E. (2000) Long-term effects of vitamin E, vitamin C, and combined supplementation on urinary 7-hydro-8-oxo-2'-deoxyguanosine, serum cholesterol oxidation products, and oxidation resistance of lipids in nondepleted men. Arterioscler Thromb Vasc Biol 20: 2087-2093.

PRINCEN, H., POST, S., TWISK, J. (1997) Regulation of bile acid biosynthesis. Curr Pharm Design 3: 59-84.

PRZYGONSKI, K., JELEN, H., WASOWICZ, E. (2000) Determination of cholesterol oxidation products in milk powder and infant formulas by gas chromatography and mass spectrometry. Nahrung. 44: 122-125.

RAMAKRISHNAN U. (2002) Prevalence of micronutrient malnutrition worldwide. Nutr Rev 60: S46-52.

RAO, M.S., REDDY, J.K. (1987) Peroxisome proliferation and hepatocarcinogenesis. Carcinogenesis 8: 631-636.

REHBERG, E.F., SAMSON-BOUMA, M.E., KIENZLE, B., BLINDERMAN, L., JAMIL, H., WETTERAU, J.R., AGGERBECK, L.P., GORDON, D.A. (1996) A novel abetalipoproteinemia genotype. Identification of a missense mutation in the 97-kDa subunit of the microsomal triglyceride transfer protein that prevents complex formation with protein disulfide isomerase. J Biol Chem. 271: 29945-29952.

REID, V.C., MITCHINSON, M.J. (1993) Toxicity of oxidised low density lipoprotein towards mouse peritoneal macrophages in vitro. Atherosclerosis 98: 17-24.

RENNERT, H., FISCHER, R.T., ALVAREZ J.G., TRZASKOS, J.M., STRAUSS, J.F. (1990) Generation of regulatory oxysterols: 26-hydroxylation of cholesterol by ovarian mitochondria. Endocrinology 127: 738-746.

RICCI, B., SHARP, D., O'ROURKE, E., KIENZLE, B., BLINDERMAN, L., GORDON, D., SMITH-MONROY, C., ROBINSON, G., GREGG, R.E., RADER, D.J., ET, A.L. (1995) A 30-amino acid truncation of the microsomal triglyceride transfer protein large subunit disrupts its interaction with protein disulfide-isomerase and causes abetalipoproteinemia. J Biol Chem 270: 14281-14285.

RIDGWAY, N.D. (1995) 25-Hydroxycholesterol stimulates sphingomyelin synthesis in Chinese hamster ovary cells. J Lipid Res 36: 1345-1358.

RINGSEIS, R., EDER, K. (2002) Insufficient dietary vitamin e increases the concentration of 7beta-hydroxycholesterol in tissues of rats fed salmon oil. J Nutr 132: 3732-3735.

RINGSEIS, R., EDER, K. (2003) Effects of dietary fish oil and oxidized cholesterol on the concentration of 7β-hydroxycholesterol in liver, plasma, low density-lipoprotein and erythrocytes of rats at various vitamin E supply. E J Lipid Sci Technol (im Druck).

RONG, J.X., RANGASWAMY, S., SHEN, L., DAVE, R., CHANG, Y.H., PETERSON, H., HODIS, H.N., CHISOLM, G.M., SEVANIAN, A. (1998) Arterial injury by cholesterol oxidation products causes endothelial dysfunction and arterial wall cholesterol accumulation. Arterioscler Thromb Vasc Biol 18: 1885-1894.

RONG, J.X., SHEN, L., CHANG, Y.H., RICHTERS, A., HODIS, H.N., SEVANIAN, A. (1999) Cholesterol oxidation products induce vascular foam cell lesion formation in hypercholesterolemic New Zealand white rabbits. Arterioscler Thromb Vasc Biol 19: 2179-2188.

ROONEY, M.W., YACHNIN, S., KUCUK, O., LIS, L.J., KAUFFMAN, J.W. (1985) Oxygenated cholesterols synergistically immobilize acyl chains and enhance protein helical structure in human erythrocyte membranes. Biochim Biophys Acta 820: 33-39.

RUEF, J., PETER, K., NORDT, T.K., RUNGE, M.S., KUBLER, W., BODE, C. (1999) Oxidative stress and atherosclerosis: ist relationship to growth factors, thrombus formation and therapeutic approaches. Thromb Haemost 82 Suppl 1: 32-37.

RUIZ-GUTIERREZ, V., PEREZ-ESPINOSA, A., VAZQUEZ, C.M., SANTA-MARIA, C. (1999) Effects of dietary fats (fish, olive and high-oleic-acid sunflower oils) on lipid composition and antioxidant enzymes in rat liver. Br J Nutr 82: 233-241.

SAITO, M., NAKATSUGAWA, K. (1994) Increased susceptibility of liver to lipid peroxidation after ingestion of a high fish oil diet. Int J Vitam Nutr Res 64: 144-151.

SALONEN, J.T., NYYSSÖNEN, K., SALONEN, R., PORKKALA-SARATAHO, E., TUOMAINEN, T.-P., DICZFALUSY, U., BJÖRKHEM, I. (1997) Lipoprotein oxidation and progression of carotid atherosclerosis. Circulation 95: 840-845.

SANDERS, T.A., SULLIVAN, D.R., REEVE, J., THOMPSON, G.R. (1985) Triglyceride lowering effect of marine polyunsaturates in patients with hypertriglyceridemia. Atherosclerosis 5: 459-465.

SARKAR, S., YADAV, P., TRIVEDI, R., BANSAL, A.K., BHATNAGAR, D. (1995) Cadmium-induced lipid peroxidation and the status of the antioxidant system in rat tissues. J Trace Elem Med Biol 9: 144-149.

SATO, R., IMANAKA, T., TAKATSUKI, A., TAKANO, T. (1990) Degradation of newly synthesized apolipoprotein B-100 in a pre-Golgi compartment. J Biol Chem 265: 11880-11884.

SATO, R., MIYAMOTO, W., INOUE, J., TERADA, T., IMANAKA, T., MAEDA, M. (1999) Sterol regulatory element-binding protein negatively regulates microsomal triglyceride transfer protein gene transcription. J Biol Chem 274: 24714-24720.

SAUCIER, S.E., KANDUTSCH, A.A., CLARK, D.S., SPENCER, T.A. (1993) Hepatic uptake and metabolism of ingested 24-hydroxycholesterol and 24(S),25-epoxycholesterol. Biochim Biophys Acta 1166: 115-123.

SCHÄFER, H., ELMADFA, I. (1984) Relative bioactivity of alpha- and gamma-tocopherol calculated from respiration parameters in rat liver mitochondria. Ann Nutr Metab 28: 297-304.

SEN, C.K., ATALAY, M., AGREN, J., LAAKSONEN, D.E., ROY, S., HANNINEN, O. (1997) Fish oil and vitamin E supplementation in oxidative stress at rest and after physical exercise. J Appl Physiol 83: 189-195.

SEVANIAN, A., HODIS, H.N., HWANG, J., MCLEOD, L.L. PETERSON, H. (1995) Characterization of endothelial cell injury by cholesterol oxidation products found in oxidized LDL. J Lipid Res 36: 1971-1986.

SEVANIAN, A., MCLEOD, L.L. (1987) Cholesterol autoxidation in phospholipid membrane bilayers. Lipids 22: 627-636.

SHARP, D., BLINDERMAN, L., COMBS, K.A., KIENZLE, B., RICCI, B., WAGER-SMITH, K., GIL, C.M., TURCK, C.W., BOUMA, M.E., RADER, D.J., ET, A.L. (1993) Cloning and gene defects in microsomal triglyceride transfer protein associated with abetalipoproteinaemia. Nature 365: 65-69.

SHETH, S., BRITTENHAM, G.M. (2000) Genetic disorders affecting proteins of iron metabolism: clinical implications. Annu Rev Med 51: 443-464.

SHINITZKY, M. (1984) Membrane fluidity and cellular function. In: Physiology of Membrane Fluidity (Shinitzky, M., ed.), Vol. 1: 1-51. CRC Press, Bocca Raton, FL.

SHULL, S., HEINTZ, N.H., PERIASAMY, M., MANOHAR, M., JANSSEN, Y.M., MARSH, J.P., MOSSMAN, B.T. (1991) Differential regulation of antioxidant enzymes in response to oxidants. J Biol Chem 266: 24398-24403.

SIES, H. (1989) Relationship between free radicals and vitamins: an overview. In: Elevated dosages of vitamins. Walter, P., Stähelin, H., Brubacer, G. (Eds.), Hans Huber Publishers, Toronto: 215-223.

SIVARAMAKRISHNAN, M.R., PYNADATH, T.I. (1982) Increased liver oleic acid synthesis in cholesterol-fed rabbits. Atherosclerosis 41: 21-25.

SKRZEP-POLOCZEK, B., TOMASIK, A., TARNAWSKI, R., HYLA-KLEKOT, L., DYDUCH, A., WOJCIECHOWSKA, C., WESOLOWSKI, W., KOPIECZNA-GRZEBIENIAK, E., ZALEJSKA-FIOLKA, J., WIDERA, E. (2001) Nephrotic origin hyperlipidemia, relative reduction of vitamin E level and subsequent oxidative stress may promote atherosclerosis. Nephron 89: 68-72.

SMITH, L.L. (1996) Review of progress in sterol oxidations.: 1987-1995. Lipids 31: 453-487.

SMITH, L.L., JOHNSON, B.H. (1989) Biological activities of oxysterols. Free Radic Biol Med 7: 285-332.

SONG, J.H., FUJIMOTO, K., MIYAZAWA, T. (2000) Polyunsaturated (n-3) fatty acids susceptible to peroxidation are increased in plasma and tissue lipids of rats fed docosahexaenoic acid-containing oils. J Nutr 130: 3028-3033.

SPRINGER, T.A. (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76: 301-314.

STANGL, G.I., KIRCHGESSNER, M., EDER, K., REICHLMAYR-LAIS, A.M. (1994) Effect of dietary hyperlipidemic components and fish oil on concentration of lipids in liver and liver fatty acid profile of rats. Z Ernahrungswiss 33: 195-206.

STANGL, G.I., NÖSTELBACHER, K., EDER, K., KIRCHGESSNER, M. (2000) Chronic vitamin E inadequacy and thermally treated oils affect the synthesis of hepatic metallothionein isoforms. Eur J Nutr 39: 112-120.

STAPRANS, I., PAN, X.-M., RAPP, J.H., FEINGOLD, K.R. (1998) Oxidized cholesterol in the diet accelerates the development of aortic atherosclerosis in cholesterol-fed rabbits. Arterioscler Thromb Vasc Biol 18: 977-983.

STEINBERG, D. (1997a) Lewis A. Conner Memorial Lecture. Oxidative modification of LDL and atherogenesis. Circulation 95: 1062-1071.

STEINBERG, D. (1997b) Low density lipoprotein oxidation and its pathobiological significance. J Biol Chem 272: 20963-20966.

STILLWELL, W., EHRINGER, W.D., DUMAUAL, A.C., WASSALL, S.R. (1994) Cholesterol condensation of alpha-linolenic and gamma-linolenic acid-containing phosphatidylcholine monolayers and bilayers. Biochim Biophys Acta 1214: 131-136.

STREULI, R.A., KANOFSKY, J.R., GUNN, R.B., YACHNIN, S. (1981) Diminished osmotic fragility of human erythrocytes following the membrane insertion of oxygenated sterol compounds. Blood 58: 317-325.

SUAREZ, A., RAMIREZ-TORTOSA, M., GIL, A., FAUS, M.J. (1999) Addition of vitamin E to long-chain polyunsaturated fatty acid-enriched diets protects neonatal tissue lipids against peroxidation in rats. Eur J Nutr 38: 169-176.

SULYOK, S., BAR-POLLAK, Z., FEHER, E., KEMENES, I., KANTOR, I., FEHER, J. (1984) Liver lipid peroxidation induced by cholesterol and ist treatment with a dihydroquinoline type free radical scavenger in rabbits. Acta Physiol Hung 64: 437-442.

SZOSTEK, R., KUCUK, O., LIS, L.J., TRACY, D., MATA, R., DEY, T., KAUFFMAN, J.W., YACHNIN, S., WESTERMAN, M.P. (1991) Effect of inserted oxysterols on phospholipid packing in normal and sickle red blood cell membranes. Biochem Biophys Res Commun 180: 730-734.

TAKAGI, A., SAI, K., UMEMURA, T., HASEGAWA, R., KUROKAWA, Y. (1992) Hepatomegaly is an early biomarker for hepatocarcinogenesis induced by peroxisome proliferators. J Environ Pathol Toxicol Oncol 11: 145-149.

TAKAHASHI, M., TSUBOYAMA-KASAOKA, N., NAKATANI, T., ISHII, M., TSUTSUMI, S., ABURATANI, H., EZAKI, O. (2002) Fish oil feeding alters liver gene expression to defend against PPARα activation and ROS production. 282: G338-G348.

TAKASHI, I., HIDEYKI, K., LAKSHMIKTTYAMMA, A., ROUYER, I.E., YOKO, T., TOSHIFUMI, A., TAKASHI, H., MICHINAO, M. (2000) Comparative effects of perilla and fish oils on the activity and gene expression of fatty acid oxidation enzymes in rat liver. Biochim Biophys Acta 1485: 23-35.

TENG, J.I., SMITH, L.L. (1995) High-performance liquid chromatographic analysis of human erythrocyte oxysterols as $\Delta^4$-3-ketone derivatives. J Chromatogr A 691: 247-254.

TENG, J.I., SMITH, L.L. (1973) Sterol metabolism. XXIV. On the unlikely participation of singlet molecular oxygen in several enzyme oxygenations. J Am Chem Soc 95: 4060-4061.

TERAO, J., SUGINO, K., MATSUSHITA S. (1985) $Fe^{2+}$ and ascorbic acid induced oxidation of cholesterol in phosphatidylcholine liposomes and its inhibition by alpha-tocopherol. J Nutr Sci Vitaminol 31: 499-508.

THEUNISSEN, J.J., JACKSON, R.L., KEMPEN, H.J., DEMEL, R.A. (1986) Membrane properties of oxysterols. Interfacial orientation, influence on mebrane permeability and redistribution between membranes. Biochim Biophys Acta 860: 66-74.

THOMAS, J.P., MAIORINO, M., URSINI, F., GIROTTI, A.W. (1990) Protective action of phospholipid hydroperoxide glutathione peroxidase against membrane-damaging lipid peroxidation. J Biol Chem 265: 454-461.

TOLBERT, N.E. (1981) Metabolic pathways in peroxisomes and glyoxysomes. Annu Rev Biochem 50: 133-157.

TOMOYORI, H., CARVAJAL, O., NAKAYAMA, M., KISHI, T., SATO, M., IKEDA, I., IMAIZUMI, K. (2002) Lymphatic transport of dietary cholesterol oxidation products, cholesterol and triacylglycerols in rats. Biosci Biotechnol Biochem 66: 828-834.

TOYOKUNI, S. (1996) Iron-induced carcinogenesis: the role of redox regulation. Free Radic Biol Med 20: 553-566.

TWISK, J., GILLIAN-DANIEL, D.L., TEBON, A., WANG, L., BARRETT, P.H.R., ATTIE, A.D. (2000) The role of the LDL receptor in apolipoprotein B secretion. J Clin Invest 105: 521-532.

UPSTON, J.M., WITTING, P.K., BROWN, A.J., STOCKER, R., KEANEY, J.F.JR. (2001) Effect of vitamin E on aortic lipid oxidation and intimal proliferation after arterial injury in cholesterol-fed rabbits. Free Radic Biol Med 31: 1245-1253.

URSINI, F., MAIORINO, M., SEVANIAN, A. (1991) in Oxidative Stress: Oxidants and Antioxidants (Sies, H., Ed.) pp 319-336, Academic Press, New York.

VAN DE BOVENKAMP, P., KOSMEIJER-SCHUIL, T.G., KATAN, M.B. (1988) Quantification of oxysterols in Dutch foods: egg products and mixed diets. Lipids 23: 1079-1085.

VAN DER ZEE, J., KROOTJES, B.B.H., CHIGNELL, C.F., DUBBELMAN, T.M.A.R., VAN STEVENINCK, J. (1993) Hydroxyl radical generation by a light-induced Fenton reaction. Free Radic Biol Med 14: 105-113.

VARGAS, R.E., ALLARD, J.B., BIGGERT, M.D., NABER, E.C. (1986) Effect of dietary 7-ketocholesterol, pure, or oxidized cholesterol on hepatic 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity, energy balance, egg cholesterol concentration, and 14C-acetate incorporation into yolk lipids of laying hens. Poult Sci 65: 1333-1342.

VATASSERY, G.T., QUACH, H.T., SMITH, W.E., KRICK, T.P., UNGAR, F. (1997a) Analysis of hydroxy and keto cholesterols in oxidized brain synaptosomes. Lipids 32: 101-107.

VATASSERY, G.T., QUACH, H.T., SMITH, W.E., UNGAR, F. (1997b) Oxidation of cholesterol in synaptosomes and mitochondria isolated from rat brains. Lipids 32: 879-886.

VAYA, J., AVIRAM, M., MAHMOOD, S., HAYEK, T., GRENADIR, E., HOFFMAN, A., MILO, S. (2001) Selective distribution of oxysterols in atherosclerotic lesions and human plasma lipoproteins. Free Radic Res 34: 485-497.

VENKATRAMAN, J.T., ANGKEOW, P., SATSANGI, N., FERNANDES, G. (1998) Effects of dietary n-6 and n-3 lipids on antioxidant defense system in livers of exercised rats. J Am Coll Nutr 17: 586-594.

VENTURA, M.A., WOOLLETT, L.A., SPADY, D.K. (1989) Dietary fish oil stimulates hepatic low density lipoprotein transport in the rat. J Clin Invest 84: 528-537.

VINE, D.F., CROFT, K.D., BEILIN, L.J, MAMO, J.C.L. (1997) Absorption of dietary cholesterol oxidation products and incorporation into rat lymph chylomicrons. Lipids 32: 887-893.

VINE, D.F., MAMO, J.C.L., BEILIN, L.J., MORI, T.A., CROFT, K.D. (1998) Dietary oxysterols are incorporated in plasma triglyceride-rich lipoproteins, increase their

susceptibility to oxidation and increase aortic cholesterol concentration of rabbits. J Lipid Res 39: 1995-2004.

WANG, S.-L., DU, E.Z., MARTIN, T.D., DAVIS, R.A. (1997) Coordinate regulation of lipogenesis, the assembly and secretion of apolipoprotein B-containing lipoproteins by sterol response element binding protein 1. J Biol Chem 272: 19351-19358.

WANG, X., BRIGGS, M.R., HUA, X., YOKOYAMA, C., GOLDSTEIN, J.L., BROWN, M.S. (1993) Nuclear protein that binds sterol regulatory element of low density lipoprotein receptor promoter. II. Purification and characterization. J Biol Chem 268: 14497-14504.

WANG, X., SATO, R., BRWON, M.S., HUA, X., GOLDSTEIN, J.L. (1994) SREBP-1, a membrane bound transvription factor released by sterol-regulated proteolysis. Cell 77: 53-62.

WETTERAU, J.R., AGGERBECK, L.P., BOUMA, M.E., EISENBERG, C., MUNCK, A., HERMIER, M., SCHMITZ, J., GAY, G., RADER, D.J., GREGG, R.E. (1992) Absence of microsomal triglyceride transfer protein in individuals with abetalipoproteinemia. Science 258: 999-1001.

WETTERAU, J.R., ZILVERSMIT, D.B. (1986) Localization of intracellular triacylglycerol and cholesteryl ester transfer activity in rat tissues. Biochim Biophys Acta 875: 610-617.

WILSON, A.M., SISK, R.M., O'BRIEN, N.M. (1997) Modulation of cholestane-3 beta,5 alpha,6 beta-triol toxicity by butylated hydroxytoluene, alpha-tocopherol and beta-carotene in newborn rat kidney cells in vitro. Br J Nutr 78:479-492.

WISEMAN, S.A., VAN DEN BOOM M.A., DE FOUW, N.J., WASSINK, M.G., OP DEN KAMP, J.A., TIJBURG, L.B. (1995) Comparison of the effects of dietary vitamin E on in vivo and in vitro parameters of lipid peroxidation in the rabbit. Free Radic Biol Med 19: 617-626.

WITTING, L.A., HORWITT, M.K. (1964) Effect of degree of fatty acid unsaturation in tocopherol deficiency-induced creatinuria. J Nutr 82: 19-33.

WOOD, W.G., IGBAVBOA, U., RAO, M., SCHROEDER, F., AVDULOV, N.A. (1995) Cholesterol oxidation reduces $Ca^{2+}$ + $Mg^{2+}$-ATPase activity, interdigitation, and increases fluidity of brain synaptic plasma membranes. Brain Res 683: 36-42.

WU, W.H., MEYDANI, M., MEYDANI, S.N., BURKLUND, M.P., BLUMBERG, J.B., MUNRO, H.N. (1990) Effect of dietary iron overload on lipid peroxidation,

prostaglandin synthesis and lymphocyte proliferation in young and old rats. J Nutr 120: 280-289.

YAN, P.S. (1999) Cholesterol oxidation products. Their occurrence and detection in our foodstuffs. In: Impact of Processing on Food Safety. Jackson et al. (Ed.). Kluwer Academic/Plenum Publishers, New York, 79-98.

YAQOOB, P., SHERRINGTON, E.J., JEFFERY, N.M., SANDERSON, P., HARVEY, D.J., NEWSHOLME, E.A., CALDER, P.C. (1995) Comparison of the effects of a range of dietary lipids upon serum and tissue lipid composition in the rat. Int J Biochem Cell Biol 27: 297-310.

YEO, Y.K., HOLUB, B. (1990) Influence of dietary fish oil on the relative synthesis of triacylglycerol and phospholipids in rat liver in vivo. Lipids 25: 811-814.

YOSHIDA, K., KANEKO, K., MIYAJIMA, H., TOKUDA, T., NAKAMURA, A., KATO, M., IKEDA, S. (2000) Increased lipid peroxidation in the brains of aceruloplasminemia patients. J Neurol Sci 175: 91-95.

ZHOU, Q., JIMI, S., SMITH, T.L, KUMMEROW, F.A. (1991) The effect of 25-hydroxycholesterol on accumulation of intracellular calcium. Cell Calcium 12: 467-476.

ZHOU, Q., WASOWICZ, E., HANDLER, B., FLEISCHER, L., KUMMEROW, F.A. (2000) An excess concentration of oxysterols in the plasma is cytotoxic to cultured endothelial cells. Atherosclerosis 149: 191-197.

ZIEDEN, B., KAMINSKAS, A., KRISTENSON, M., KUCINSKIENE, Z., VESSBY, B., OLSSON, A.G., DICZFALUSY, U. (1999) Increased plasma 7β-hydroxycholesterol concentrations in a population with a high risk for cardiovascular disease. Arterioscler Thromb Vasc Biol 19: 967-971.

ZSIGMOND, E., PARRISH, C., FONG, B., ANGEL, A. (1990) Changes in dietary lipid saturation modify fatty acid composition and high-density-lipoprotein binding of adipocyte plasma membrane. Am J Clin Nutr 52: 110-119.

ZUNIN, P., EVANGELISTI, F., CABONI, M.F., PENAZZI, G., LERCKER, G., TISCORNIA, E. (1995) Cholesterol oxidation in baked foods containing fresh and powdered eggs. J Food Sci 60: 913-916.

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## *Erklärung*

Hiermit versichere ich an Eides Statt, dass ich die eingereichte Dissertation „*Nutritive Beeinflussung der Oxysterolkonzentrationen durch Fischöl, Vitamin E, Eisen und oxidiertes Cholesterin sowie ausgewählte physiologische Wirkungen oxidierten Cholesterins bei der Ratte*" selbständig angefertigt und diese nicht bereits für eine Promotion oder ähnliche Zwecke an einer anderen Universität eingereicht habe. Ferner versichere ich, dass ich die zur Erstellung der Dissertationsschrift verwendeten wissenschaftlichen Arbeiten und Hilfsmittel genau und vollständig angegeben habe.

Desweiteren erkläre ich, dass keine Strafverfahren gegen mich anhängig sind.

Halle, den 25.03.2003

Robert Ringseis