【LSP(脂質標準化プログラム)の発展に貢献した組織】

LSP(脂質標準化プログラム)のその後の発展には，疫学・臨床医学・分析化学を専門とする学術組織が大きな貢献をすることになった。1957年，心・血管病に関する統合研究会は，心・血管病の研究促進に果たす分析化学の重要性に関する所見を表明した。その所見の中で，分析室内部及び分析室間におけるコレステロールの測定値の再現性と信頼性をもっと向上させる必要があるとの答申がされた。統合研究会は，標準化や品質管理や正常値に関わる問題点を調査・研究するための事務局と基準分析室を設立することを勧告した。この勧告を受けて，コレステロールの測定に関して，1)コレステロールの純品や標準血清(凍結血清，もしくは，凍結乾燥血清)の製作の可能性，2)分析室内部及び施設間における測定値の再現性や信頼性に関する実態調査，3)濃度既知標品の供給の可能性などについて，検討することが決定された。

統合研究会の分科会(会長: Dr. Thomas R. Dawber, Dr. Dawber は Framingham Heart Disease Epidemiology Study の指導者であるとともに，National Heart Institute の代表も勤めた)は，心・血管病を研究する複数の疫学研究機関の検査室を対象にコレステロールの標準化を実施するに当たって，CDC が中立的な役割を果たす分析室として機能することを求めてきた。その理由は，それまでに疫学研究をしている研究者間で血清をお互いに交換して測定し，相互比較をしたところ，測定値にかなりの不一致が認められたことによる。個々の分析室は，皆が自分の測定値が一番正しいと主張して譲らなかった。このような雰囲気の中では，医師も分析者も標準化に同意することは難しい状況にあった。

1958年9月，別の組織である Research Committee of International Society of Cardiology は，コレステロールの標準化の必要性を説き，その品質管理と標準品による正確度の確保を図るための国際センターのような組織の設立を求めた。同年10月，WHO の Expert Committee on Cardiovascular Diseases and Hypertension は，コレステロールの標準化に向けて支援する用意のあることを表明した。翌年の春までに，ニュージャージー州のプリンストンにある Subcommittee on Biochemical Measurements of Conference on Methodology in Epidemiologic Studies of Cardiovascular Diseases は，心・血管病に関する統合研究を成功させるためには，次のような二つの手段が重要であることを繰り返し訴えていた。その二つとは，① 血清コレステロールの標準化を実施するための方法論の開発，と，② 脂質標準化の事務局と基準分析室の設立，であった。

【CDCが標準化を担当】

1957年，CDC はコレステロールの基準分析法の確立とその事務局を開設した。委員は，分析化学が専門の Dr. Eloise Eavenson，医師の Dr. A. John Schneider，統計学を専門とする Dr. Myron Willis，そして医師の Dr. Gerald Cooper で構成されていた。委員達の目標は，コレステロールの標準血清と CDC Cooperative Cholesterol Standardization Program(CCSP，コレステロール標準化プログラム)を開発する点にあった。そこで取り上げられた問題点は，1)凍結乾燥血清が果たしてコレステロールの標準血清として使用出来るかどうか，2)一次標準としての高純度のコレステロールを精製出来るかどうか，3)分析室間で発生するコレステロールの測定値の実態調査とその対策，などであった。事務局の役割は，各種濃度の凍結乾燥血清を準備して，それを各地の分析室に送付し，得られた測定成績を解析・評価して，報告することにあった。

【CDC による総コレステロールの標準化プログラムの提供】

1958年，CDC は7ヵ所の心・血管病に関する疫学研究機関に対して，最初の総コレステロールの標準化を開始した。その7施設とは，アルバニー大学(Dr. J Doyle)，ミシガン大学(Dr. F Epstein)，シカゴ衛生部(Dr. J Stamler)，ジョージア公衆衛生局(Dr. G Barrow)，フラミンガム研究組織(Dr. T Dawber)，ミネソタ大学(Dr. A Keys)，米国海軍病院(A Graybiel 大佐)であった。標準化は次のような3段階(Part)に分けて実施された。第1段階(Part 1)は，標準化に習熟することと分析室で得られた二重測定値の再現性の確認，第2段階(Part 2)は精密度と正確度の確認と技術のより一層の練磨，第3段階(Part 3)は4種類の濃度未知ヒト血清の分析，であった。当時はまだ基準分析法が確立していなかったので，この標準化の目標は精密度と参加施設間差の実態を

把握する点にあった。第1回目の標準化における測定値の標準偏差の平均値は，10.6mg/dLを記録した。参加7施設の中，6施設がAbell-Kendall法で測定し[2]，残る1施設は塩化第二鉄法で測定した[3]。その後も，CDCの標準化分析室は，1)コレステロールの標準血清として何が適切であるのか，2)高純度のコレステロール標品をどのようにして精製するのか，3)コレステロールの基準分析法としては何が適当であるのか，という点についての調査・研究を継続した。第1の点については，WHOの勧告に従って，3mL容量のガラスバイアルに4濃度の凍結乾燥血清1mLを入れたものが準備された。個々のバイアルには4桁の番号を付けたラベルが貼られ，血清を凍結乾燥処理してから，窒素ガスを充填し，アルミカバーで封印された。第2の点については，一次標準としての高純度のコレステロールの精製に関する研究が開始された。コレステロールの純品を入手するためにFieser法が採用され，20回にも及ぶ結晶化が行われた[4]。1968年から1969年にかけて，10種類のコレステロール標品を対象に，ガスクロマトグラフ，Liebermann-Burchard反応試験，薄層クロマトグラフ，赤外線分光光度計，融点測定などが実施された[5]。第3の点については，CDCの標準化分析室は5種類の分析法を取り上げ，これらの分析法がどの程度，臨床検査法，あるいは，基準分析法として適切であるのかという研究を開始した[6]。その5種類の分析法として候補に挙がったのは，次の5つであった。

① CDC Modified A-K method：アベルーケンダル原法をCDCで改良工夫された方法で，試薬として酢酸・無水酢酸・硫酸を使用する定量法。疫学研究機関で多く採用されており，再現性が良好とされた[2]。
② 塩化第二鉄を使用する自動分析法[7]。
③ 塩化第二鉄を使用する用手法。本法は，②法と比較して感度に優れ，より多量の検体処理が可能であるとされた[3]。
④ Tschugaeff反応を応用したHanel-Dam法。微量の検体を用いて，高感度と呈色の安定性に優れているとされた[8]。
⑤ Sperry-Webbによる Brown改良法。本法は，ジギトニンを使用する方法であるが，基準分析法に成り得ると考えられていた[9]。

これらの5種類の測定法に対する評価と比較実験は，3段階の過程を経て実施された[6]。第一段階では，分析担当者がこれらの5種類の測定法に習熟してから，再現性の検討に入った。第二段階では，5つの測定法に内在する問題点をピックアップして，それらを除去した。最終段階では，正常血清と異常血清を使用して，8週間にわたって比較・評価テストが行われた。その結果，精密性に最も優れていたのは⑤のジギトニンを使用するSperry-Webb法[6]，次いで，①のAK法と②の自動分析法の両法であった。平均19%もの高値を示した塩化第二鉄法を除いて，ジギトニン法・AK法・自動分析法の3法の再現性は比較的に小さい値を示した。血漿中のコレステロール値は血清よりも低い値をとるが，これは抗凝固剤による影響である。結果的にみて，CV値が1%以下を示し，最も問題点が少なかったAK法がコレステロール標準化プログラム(CCSP)の基準分析法として選択された。

【National Heart Instituteとの共同作業】

1961年，国立心臓研究所(NHI, National Heart Institute)の所長のDr. Jim Wattは，NHIの財政補助を受けて実施された心臓病の研究が今ひとつ成果を見ない原因は，コレステロールの測定値に疑問があるからだという結論に達した。その結果，CDCとNHIの両機関が共同してHeart Disease Control Cholesterol Laboratoryを設立し，コレステロール標準化プログラム(CCSP)を稼動させて，NHIの助成を受けた全ての疫学研究機関の検査室が利用出来るようにするということになった。1965年までには，全米で少なくとも65の疫学関係の分析室が，また，外国では18の疫学研究室がコレステロール標準化プログラム(CCSP)に参加して，標準化を受けた。1962年から1965年までの間に，1000以上の臨床検査室が標準化プログラムに参加したいという希望を寄せてきたことは，興味深いことであった。しかしながら，標準化の第一段階であるPart 1の検体を受け取って報告をしてしまうと，その後は途端に参加しなくなってしまった。その理由を調査すると，これらの臨床検査室は「まあまあそこそこのデータが出ているのかどうか」という程度のことが判れば，それで十分なのだという返事であった。このような経験を踏まえて，CDCの標準化プログラムに参加できる分析室は，疫学研究に従事している機関，NHIやWHOから助成を受けている組織，公衆衛生

関連の組織，脂質に関する専門の研究機関などに限定することになった。

【WHOの協力機関に認定】

1962年，コレステロールの標準化活動を実践的に行うCDCは，WHOの協力センター(WHO Collaborating Center for Reference and Research in Blood Lipids)に認定された。

その目的は，心・血管病の疫学研究に携わっていて，WHOの研究を支援している全世界の研究機関にCDCの脂質標準化プログラム(LSP)を公正・公平に利用出来るようにすることにあった。その結果，毎年約30の研究機関がLSPに参加するようになった。参加施設の中には，米国以外では，オーストラリア，オーストリア，ベルギー，中国，フランス，ドイツ，イタリア，日本，オランダ，ニュージーランド，ポルトガル，スペイン，チェコスロバキア，及び，イギリスの各国が含まれている。

【標準化による成果】

コレステロール標準化プログラム(CCSP)の効果に関する1966年の報告書によれば，147mg/dL，186mg/dL，226mg/dL，267mg/dLから成る4濃度の血清を測定したPart 3終了の分析室14施設の測定精度の中，コレステロールの正確度はAK法で測定した検査室のバイアスがCDCの目標値に対して＋4mg/dL，LB直接法とPearson法が共に＋25mg/dL，塩化第二鉄法が＋14mg/dLといずれも高めの値を示し，一方，精密度はどの測定法でも変動係数で2～4%を示した。

【トリグリセライド】

1966年から1968年にかけて，CDCは19施設の分析室を対象に，トリグリセライドの標準化プログラムを実験的に実施した。この試行プログラムはDr. WitterとMs. Whitnerにより考案されたものである。実験の目標は，各種の測定法でトリグリセライドを測定した時，どの程度の精密さや正確さが得られるのか，という点を把握することにあった。先ず，トリグリセライドの基準分析法としてWake Forest大学のDr. Loflandと共同開発された測定法，即ち，ケイ酸とクロロホルムで血清を前処理して，抽出液を蒸発乾固後にアルコール性水酸化カリウムで加水分解し，生成したグリセロールをメタ過ようそ酸ナトリウムと砒素化合物で酸化分解し，酸化物をクロモトロープ酸と濃硫酸で加温縮合反応させて，比色定量する方法が採用された[10]。一次標準物質の違いによって測定値の表現に差が出ることを避けるために，測定値はmmol/Lで報告された。測定成績からみて，トリグリセライドの許容範囲は正確度で＋/－0.1 mmol/L，精密度ではCVで5%と推定された。

1968年，トリグリセライドの標準化プログラムが105施設を対象に実施された。このプログラムは，総コレステロールのプログラムと類似していた。標準化参加分析室は，1.11mmol/Lから1.74mmol/Lまでの濃度分布をする20検体を二重測定した。これら105施設の中には，Part 1からPart 3までの各段階の標準化検査室が含まれているが，その測定精度は，39施設が精密度・正確度共に優れ，29施設が精密度は良好であるが，正確度が不良，13施設が正確度は良好であるが，精密度が不良，24施設は精密度・正確度共に不良という状況であった。1974年には，総コレステロールとトリグリセライドの両標準化プログラムが，一本化されてCooperative Cholesterol and Triglyceride Standardization Program (CCTSP，コレステロール・トリグリセライド共通標準化プログラム)となった。この標準化では，同一血清を使って総コレステロールとトリグリセライドの2項目が同時に標準化された。凍結血清と凍結乾燥血清共に，－20℃での総コレステロールとトリグリセライドの安定性には問題は認められなかった[11]。

【HDLコレステロールの登場】

1970年代になって，心・血管病の危険因子としてのHDLコレステロールの果たす役割についての医学的関心が，急速に盛り上がった。1981年，その重要性からHDLコレステロールの標準化が，これまでのLSP(脂質標準化プログラム)に追加されることになった。HDLコレステロールの基準分析法は，超遠心法でVLDLを除去してから，上清中のLDLをヘパリン・マンガン法で沈殿分離し，その後アベル・ケンダル法(A-K)でHDLコレステロールを定量する方法である。本法に関する研究によれば，①HDLコレステロールはイオン強度により強い影響を受けること，②血清を1年以上安定的に保存するためには－70℃で冷凍する必要性があること，③低

濃度のHDLコレステロールの再現性がかなり大きいこと，などが指摘されている。

【LSP(脂質標準化プログラム)のその後の拡張と発展】

1984年，LSP(脂質標準化プログラム)は，これまでの総コレステロールとトリグリセライドにHDLコレステロールの標準化を加え，CDC-NHLBI Lipid Standardization Programと改名された。LSPには，100以上の施設の参加がある。例えば，2001年現在では，米国で70施設が，また，米国以外の外国では31施設が参加している。LSPを通じて，CDCはこれまでに4つの心・血管病に関する大規模疫学研究における中央検査室としての役割を担ってきた。1963年から1965年にかけて実施されたNational Diet-Heart Studyでは，CDCはコレステロールの分析を担当したが，この研究では検体は全米の6施設から送られてきた。1965年から1975年にかけて実施されたCoronary Drug Projectでは，CDCは多項目の血液化学検査における中央検査室としての役割を果たした。この時の研究では，臨床から53施設の参加があった。1970年から1972年にかけて実施されたCooperative Lipoprotein Phenotyping Studyでは，脂質及びリポ蛋白の分析における中央分析室として機能した。CDCは，また，米国政府による3回の国民栄養調査，即ち，HANES I(National Health and Nutrition Examination Survey, 1971～1975)，HANES II(1976～1980)，及び，Hispanic HANES(1982～1984)における中央検査室としての役割を果たしてきた。CDC-NHLBI脂質標準化プログラムは，これまでに30以上の米国内，並びに，国際的な臨床試験や疫学研究を支援してきた。その中には，Lipid Research Clinics(LRC)，Multiple Risk Factor Intervention Trial(MRFIT)，Program on Surgical Control of Hyperlipidemia，Air Force Health Evaluation Risk and Tabulation Programというような大型プロジェクトが含まれている。

1975年，米国臨床化学会(American Association for Clinical Chemistry, AACC)は，コレステロールの基準分析法に関する研究会を設立した[12]。この研究会は，基準分析法としてCDCのアベル・ケンダル(AK)法と酵素法の両分析法を候補として推薦した[13]。委員会は，二つの分析法のどちらが二次標準としての実用基準分析法としてより適切であるのかという点について検討を始めると同時に，NIST(National Institute of Standards and Technology)が一次標準としての絶対基準分析法の開発を担当することになった。その後の厳密な検証を経て[12]，最終的にCDCのアベル・ケンダル(AK)法が実用基準分析法として，また，アイソトープ希釈質量分析法(Isotope Dilution Mass Spectrometry, IDMS)が絶対基準分析法として採用された[14]。1990年から2002年の間に，107.1mg/dLから345.4mg/dLの濃度域を持つ53種類のコレステロール用標準血清を12回(1回の測定では，二重測定を4回実施。従って，計96個の測定値を元に計算)に分けて分析したところ，CDCのアベル・ケンダル(AK)法では精密度がCVで0.43%(Within-run CVが0.35%，Among-run CVが0.23%)，正確度ではIDMS法に比べて＋1.6%高い値を示した。アベル・ケンダル(AK)法による測定値の方が高めになる理由は，主として血清中に存在するステロールの先駆物質やPhytosterolsやコレステロールの酸化物質によるものである[15]。以上のような経緯を経て，CDCで改良されたアベル・ケンダル(AK)法が，NCCLS(National Committee for Clinical Laboratory Standards)の臨床検査委員会によって，米国におけるコレステロールの正式の基準分析法として採択されるに至ったのである。米国におけるコレステロールの基準システム(National Reference System)は，CDCの実用基準分析法としてのアベル・ケンダル(AK)法，NISTの絶対基準分析法としてのIDMS法，NISTによるコレステロールの一次標準物質としてのSRM 911，そして，CDCで製造されNISTから供給されている標準血清から構成されている[16]。CDCは，NHLBIと共同して国民向けのコレステロール教育プログラム(National Cholesterol Education Program, NCEP)の確立に努力した。このNCEPでは，総コレステロールの正確度はCDCの目標値の±3%以内，精密度は変動係数で3%以下を設定することによって，心・血管病の危険因子別に臨床的に正しく分類することが求められている[17]。1993年，NCEPのリポ蛋白に関する作業部会は，測定精度の許容限界として，LDLコレステロールでは正確度は基準分析法の目標値の±4%以内，精密度はCVで4%以下を[18]，HDLコレステロールでは正確度は±5%以内，精密度はCVで4%以下を[19]，また，トリグリセライドでは正確度は±5%以内，精密度はCV

で5%以下であることを勧告している[20]。CDCの脂質標準化プログラム(LSP)は，このNCEPの勧告を受け入れて実践活動が行われている。現在，CDCの脂質標準化グループの最高責任者はDr. Gary Myers，Cholesterol Reference Method laboratory Network(CRMLN)の総括責任者はDr. Mary Kimberly，基準分析室の分析責任者はDr. Parvin Waymack，筆者のGerald R. Cooper，MD，PhDはCDCの脂質標準化活動における医学顧問を担当している。

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Original Articles

Improvement in Japanese Clinical Laboratory Measurements of Total Cholesterol and HDL-cholesterol by the US Cholesterol Reference Method Laboratory Network

Masakazu Nakamura, Shinichi Sato, and Takashi Shimamoto

Lipid Reference Laboratory, Osaka Medical Center for Health Science and Promotion, Osaka, Japan.

Background: Accurate and precise measurements of total cholesterol (TC) and HDL-cholesterol (HDL-C) are necessary for effective diagnosis and treatment of lipid disorders. We studied the impact of TC certification and HDL-C evaluation in Japanese clinical laboratories to standardize their measurements.

Methods: We selected 78 laboratories participated at least twice for TC and 46 laboratories participated twice for HDL-C in the standardization protocols developed by the Cholesterol Reference Method Laboratory Network (CRMLN). We compared the initial and subsequent results using the performance guidelines established by US National Cholesterol Education Program (NCEP).

Results: For TC, mean percentage bias of all participants was −0.93% and −0.49% for the initial and second rounds, respectively. Mean within-sample CV was 0.72% and 0.69% for the initial and second rounds, respectively. For HDL-C, mean percentage bias of all participants was −1.86% and −0.06% for the initial and second events, respectively. Mean among-run CV was 1.56% and 1.58% for the initial and second events, respectively.

Conclusions: TC accuracy in the second round than the initial round tended to improvement although statistically not significant, however in the five years follow-up, mean absolute percentage bias was reduced over time. HDL-C accuracy was statistically improved in the second event than the initial event. The precision for both TC and HDL-C did not change. This study shows CRMLN protocols contribute effectively to improvement of TC and HDL-C performance. ***J Atheroscler Thromb, 2003; 10: 145–153.***

Key words: Total cholesterol, HDL-cholesterol, Accuracy, Precision

Introduction

Research for epidemiological studies and clinical trials have demonstrated that high TC and/or high LDL-cholesterol (LDL-C) are an important risk factor for coronary heart disease (CHD) (1–5) and that low HDL-C is an independent predictor of risk for CHD (6–8). According to the recent studies LDL-lowering therapy robustly reduces risk for CHD (9–13).

Guidelines for diagnosis and treatment of lipid disorders were issued in three reports from the US NCEP Adult Treatment Panel (ATP) in 1988 for ATP-I, in 1993 for ATP-II and in 2001 for ATP-III (14). The European Atherosclerosis Society (EAS) issued similar guideline in 1998 (15). The Japan Atherosclerosis Society (JAS) issued the first guideline in 1997 and updated it in 2002 (16). To identify individuals at risk for CHD, the NCEP recommends initial classification in ATP-III using the medical decision points

Address for correspondence: Masakazu Nakamura, Lipid Reference Laboratory, Osaka Medical Center for Health Science and Promotion, 1-3-2 Nakamichi, Higashinari-ku, Osaka, Japan 537-0025.
E-mail: xnakamura@kenkoukagaku.jp
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of 5.17 and 6.21 mmol/L (200 and 240 mg/dL) for TC and of 1.03 mmol/L (40 mg/dL) for HDL-C, and the JAS recommends initial classification in 2002 revision using the medical cut points of 5.69 mmol/L (220 mg/dL) for TC and of 1.03 mmol/L (40 mg/dL) for HDL-C. Any recent guideline emphasizes the importance for measurement of LDL-C rather than TC.

Accurate, reproducible, and comparable measurements of TC and HDL-C are needed for effective application of the guidelines. The NCEP recommendations indicate that the performance goals for TC are accuracy, expressed as percentage bias versus the accuracy base, of $\leq \pm 3\%$ and imprecision, expressed as CV, of $\leq 3\%$ (17). The performance goals for HDL-C are accuracy, expressed as percentage bias versus the accuracy base, of $\leq \pm 5\%$ and imprecision, expressed as CV, of $\leq 4\%$ at HDL-C concentrations > 1.09 mmol/L (42 mg/dL) and as standard deviation ≤ 0.044 mmol/L (1.7 mg/dL) at HDL-C $\leq$ 1.09 mmol/L (42 mg/dL) (18, 19).

In 1990, the CDC established the CRMLN (20,21) to improve lipid and lipoprotein measurements by providing traceability to the accuracy bases for these analytes. In addition to US-based laboratories, CDC, in its role as a World Health Organization Collaborating Center for Reference and Research in Blood Lipids, extended the CRMLN to include selected international laboratories. The Osaka Medical Center for Health Science and Promotion (OMC) has been a member of the CRMLN since July 1992. The CRMLN developed a process by which manufacturers and clinical laboratories can establish traceability to the US National Reference System for Cholesterol (NRS/CHOL), as recommended by NCEP. In 1995, this process was extended to manufacturers producing products used to measure HDL-C (22).

Traceability for TC in a clinical laboratory is verified by comparing the field method with the Abell, Levy, Brodie and Kendall (AK) reference method for TC (23, 24) in a CRMLN laboratory. The comparison is performed based on the CRMLN's Certification Protocol for Clinical Laboratories (25). For HDL-C, the CRMLN has not yet established a specific protocol for certifying clinical laboratories. We therefore applied the CRMLN protocol for certifying manufacturers to Japanese clinical laboratories. The protocol for manufacturers involves a comparison between the field method and the designated comparison method (DCM) for HDL-C using a minimum of 40 to 50 fresh human specimens (26).

The impact of standardization in clinical laboratories has not been well documented. We report here the results for the effectiveness of the CRMLN's TC and HDL-C protocols toward improvement of Japanese clinical laboratory measurements. They participated in the certification process on a voluntary basis. At the same time, to know the effectiveness of long-term standardization, we also report the results based on 5 years of follow-up comparisons for TC.

Materials and Methods

Reference methods

The AK reference method (23, 24) consists of saponification of a 0.5-mL serum sample with alcoholic potassium hydroxide, extraction with hexane, evaporation of an aliquot of the extract, development of color with Liebermann-Burchard reagent at 620 nm, and calibration by the NIST SRM 911b pure cholesterol material. HDL-C DCM employs direct precipitation of the apo-B-containing lipoproteins with dextran sulfate of 50 kDa with magnesium, followed by measurement using the reference method for TC (22).

Comparison protocol for TC

Those laboratories standardizing TC methods followed the CRMLN's certification protocol for clinical laboratories. The protocol required the laboratory to collect a set of six fresh individual serum specimens, however they could combine serum up to two individual donors to obtain the necessary volume or concentrations. The following guidelines for collection were provided to the laboratories: 1) Collect two samples in each of three concentration regions: 2.59–5.17 mmol/L (100–200 mg/dL), 5.17–6.21 mmol/L (200–240 mg/dL), and > 6.21 mmol/L (> 240 mg/dL); 2) make sure the range of concentration between the lowest and the highest is at least 2.59 mmol/L (100 mg/dL); and 3) make sure at least 0.52 mmol/L (20 mg/dL) difference exist between the concentrations of samples in each of the three regions.

All participants used commercially prepared enzymatic reagents and human serum-based calibrators. The assay principle of all reagents is the cholesterol ester hydrolase-cholesterol oxidase-peroxidase chromogenic method. Specimens were analyzed in duplicate on three separate days for a total of six replicate measurements per sample. After these measurements were completed, frozen aliquots were shipped on dry ice by overnight express delivery to OMC.

Selection of laboratories for TC

Of the 291 Japanese clinical laboratories, 78 laboratories were selected for this study because they participated two or more times. Some of these laboratories were involved in an epidemiological study for the Japan Public Health Center-based prospective Study on cancer and cardiovascular diseases (JPHC Study) (27) and a clinical trial for the Pravastatin Anti-atherosclerosis Trial in the Elderly (PATE) (12, 28).

Initially, we compared the results of clinical laboratories that participated twice, and subsequently, we compared the results of laboratories that participated more

than twice during a 5-year period. During the 5 years of the study, 10 standardization rounds were conducted. Of the 78 laboratories that participated in the first two rounds, the number declined over 5 years so that only 9 of the original laboratories remained in the 10th round.

Data analysis for TC

The analysis spreadsheet calculated average percentage bias, average absolute percentage bias, average within-sample CV, within- and between-method outliers, and linear regression statistics. The regression statistics were used to calculate the bias at the medical decision points of 5.17 and 6.21 mmol/L (200 and 240 mg/dL). Laboratories meeting the following criteria were qualified to receive "Certificate of Traceability": average absolute percentage bias ≤ 3%, percentage bias at 5.17 and 6.21 mmol/L ≤ 3%, CV ≤ 3%, correlation coefficient (r^2) ≥ 0.975, and no within- or between-method outliers. The certificate is valid for 6 months.

Comparison protocol for HDL-C

Those laboratories standardizing HDL-C methods followed the CRMLN's evaluation protocol for manufacturers (26). The sample comparison is based upon the US National Committee for Clinical Laboratory Standards protocol "Method comparison and bias estimation using patient samples; approved guideline" (29). The protocol requires analysis of a minimum of 40–50 fresh patient specimens. Samples were selected with the range of HDL-C concentrations, 0.52–1.81 mmol/L (20–70 mg/dL). To achieve this goal, a minimum of five samples were collected in each of the following concentration regions: 0.52–0.75 mmol/L (20–29 mg/dL), 0.78–1.01 mmol/L (30–39 mg/dL), 1.03–1.27 mmol/L (40–49 mg/dL), 1.29–1.53 mmol/L (50–59 mg/dL), and 1.55–1.78 mmol/L (60–69 mg/dL). The remaining samples, a minimum of 15, were spread over the entire concentration range. All samples had triglyceride concentration < 2.26 mmol/L (200 mg/dL).

All participants used commercially prepared reagent kits and human serum-based calibrators. All of these methods are the "direct" methods. Not all laboratories used the same kit, but the products used were from three Japanese manufacturers: Kyowa Medex Co., Ltd., Tokyo (30); Daiichi Pure Chemicals Co., Ltd., Tokyo (31); and Wako Pure Chemical Industries, Ltd. , Osaka (32).

The fresh-frozen serum samples were prepared at OMC from fasting donors including patients and volunteers. Serum was dispensed into separate vials and frozen at – 60°C or below within 8 hours after separation. The frozen samples after check of lipoprotein electrophoresis were shipped to each participant on dry ice by overnight express delivery within 3 days of sample collection.

Clinical laboratories analyzed each sample in duplicate in one run. The total number of samples was divided among five analytical runs. Between analytical runs the samples were stored at – 60°C or below. Each laboratory analyzed its own quality control (QC) sample with HDL-C concentration of 0.78 to 1.55 mmol/L (30 to 60 mg/dL). Each laboratory used either a commercial QC product or prepared its own sample from pooled human serum. Single measurements from 20 recent analytical runs, including the runs where comparison samples were analyzed, were used to estimate among-run CV.

Selection of laboratories for HDL-C

Of the 200 Japanese clinical laboratories, 46 laboratories were selected for this study because they participated twice.

Data analysis for HDL-C

The analysis spreadsheet calculated average percentage bias, average absolute percentage bias, average within-sample within-run CV, within- and between-method outliers, and linear regression statistics. The regression statistics were used to calculate the bias at the medical decision points of 0.91 and 1.55 mmol/L (35 and 60 mg/dL). Among-run CV was also calculated from the single QC measurements with the field method. All laboratories obtained "Document of Comparison" stating that the specific analytical system had been compared with the DCM for HDL-C and listing the specific statistical parameters observed for the system. The document was valid for 2 years. For this study, the laboratories meeting the following criteria were considered to be standardized: average percentage bias ≤ 5%, percentage bias at the medical decision points of 0.91 and 1.55 mmol/L ≤ 5%, among-run CV ≤ 4%, r^2 ≥ 0.975, no more than one within-method outlier, and no between-method outliers.

Statistics

For every survey and each laboratory (e.g., first round versus second round), we calculated mean percentage bias, mean absolute percentage bias, and CV. To compare overall group mean biases and CVs on the initial and second rounds, we used the studentís *t*-test. We also calculated a *t*-statistic and *p*-value for each laboratory separately and evaluated these 78 *p*-values (33). A significance level of $\alpha = 0.05$ was used throughout this study.

In addition, for TC, we tested for reduction of bias over 10 surveys. To do this, we performed a linear weighed regression for each laboratory where the number of surveys (first to 10th) in which a laboratory participated was the independent variable and the percentage bias was the dependent variable. Weights corresponded to the inverse variances of the percentage biases obtained for each survey. Cases where the intercept is negative and the slope is positive, or vice versa, generally indicate improvement in the bias for the laboratory as more surveys are completed. We

plotted the intercepts versus the slopes for all 78 laboratories in a scatter plot. This procedure has the advantage of using all available data, rather than comparing only the first to the last surveys, for example.

Results

Standardization of TC in clinical laboratories

The average time between the first and second rounds for the 78 participating laboratories was 13 months; the median time difference was 7 months.

The performance of 78 clinical laboratories participating in the first and second rounds is presented in Table 1. Seventy-one (91.0%) and 72 (92.3%) laboratories met the performance criteria and received "Certificate of Traceability" in the first and second rounds, respectively. Overall, the pass rate in the second round did not improve significantly from the initial round.

The mean percentage bias for all laboratories improved by 0.44% between the first and second rounds, which was not statistically significant at the 95% level, but it suggests an actual difference. The mean absolute percentage bias improved by 0.20%, which did not reach statistically significance too. The mean average within-sample CV for the group of laboratories did not change.

In another evaluation of the data, we calculated the mean difference for each laboratory and formed a *t*-value for each laboratory on the basis of five degrees of freedom (from six sample means). Thus, 78 *p*-values are obtained. The median *p*-value for 78 laboratories was 0.024. Sixty-three of 78 (80.8%) were < 0.05 and statistically significant at the 95% probability level. Eighty percent of the laboratories showed significant improvement in their bias.

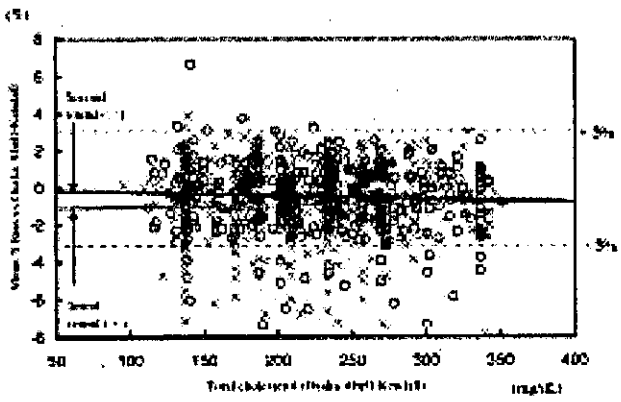

Fig. 1. Mean % Bias Plots for TC. Bias plots of 78 Japanese clinical laboratories that participated in the first and second TC certification round. Mean percentage bias is plotted versus the TC concentration (mg/dL) as determined by the AK reference method at Osaka. Horizontal dotted lines mark the NCEP bias guidelines at ± 3%. Data for all participants is presented together in this one plot. The regression line was $y = 0.0010 \times - 1.1715$ ($n = 78$, $r = 0.0284$) for the initial round (×) and $y = -0.0018 \times - 0.0626$ ($n = 78$, $r = -0.0538$) for the second round (○).

The bias of all participants in the initial and second rounds is shown in Fig. 1. This plot includes the bias of all individual samples analyzed by all 78 participants. Fewer samples had bias greater than ± 3% in the second round.

Table 1. Performance of participants in total cholesterol certification program

Round & *p*-value	Participants		Accuracy% bias		PrecisionCV
			Mean % bias (Mean ± SD)	Mean absolute % bias (Mean ± SD)	Mean within-sample CV (Mean ± SD)
Initial	All Labs	78	−0.93 ± 1.77%	1.61 ± 1.27%	0.72 ± 0.33%
	Certified[a]	71	−0.56 ± 1.31%	1.30 ± 0.72%	0.70 ± 0.34%
Second	All Labs	78	−0.49 ± 1.75%	1.41 ± 1.20%	0.69 ± 0.30%
	Certifieda	72	−0.24 ± 1.22%	1.14 ± 0.60%	0.69 ± 0.30%
p-value[b]	All Labs	78	0.11	0.31	0.65
	Certified		0.13	0.15	0.79

[a]: Only laboratories that met CRMLN performance criteria: Average percentage bias < = ± 3%, percentage bias at 5.17 and 6.21 mmol/L < = ± 3%, CV < = 3%, correlation coefficient (r^2) < = 0.975, and no within- or between-method outiers.

[b]: *p*-value for comparison of initial and second rounds

Table 2. Number of participants failing to meet specific CRMLN performance criteria for total cholesterol

Round	Accuracy	Precision	Outliers	
	Mean absolute% bias± 3%	Mean within -sample CV< = 3%	Within-method outliers	Between-method outliers
Initial	7	0	1	0
Second	5	0	1	0

In the first round of standardization, a total of seven laboratories failed to meet the criteria to obtain "Certificate of Traceablity". In the second round, a total of five laboratories failed. Table 2 summarizes the reasons that laboratories did not pass certification for both rounds of the protocol. No laboratories failed certification because of imprecision. The most common reason for failure was inaccuracy. Some laboratories failed on multiple criteria. Only one laboratory failed both rounds; this laboratory failed the first time because of inaccuracy and the second time because of within-method outliers. In a third round, this laboratory met all of the criteria.

Table 3 shows the results of 10 rounds of the TC certification protocol with the 78 laboratories that began with the initial standardization. The number of laboratories that remained in the program for all 10 rounds decreased over the 5 years of the study. However, the pass rate increased by the sixth round. Although the mean percentage bias did not appear to change significantly, the mean absolute percentage bias was reduced over time. A weighed regression of the mean absolute bias over the course of the 10 rounds (mean absolute percentage bias versus the number of surveys) had a slope of -0.050, an intercept of 1.340, and a p-value of 0.0008. This shows that the mean absolute percentage bias has been reduced significantly over the 10 surveys for these laboratories.

Standardization of HDL-C in clinical laboratories

The average period between the first and second events in the 46 participants was 20 months.

The initial and second performances of HDL-C by all participants is presented in Table 4. As a group, these laboratories' performance improved between the first and second events. For mean percentage bias and mean absolute percentage bias, differences between the initial and second events were statistically significant (p = 0.003 and p = 0.00002, respectively). Differences in the mean among-run CV between the first and second events were not statistically significant (p = 0.88). The percentage bias of all participants is shown in Fig. 2A and Fig. 2B for the first and second events, respectively.

Table 3. Trends in total cholesterol performance of certified laboratories

Round	Participants # (remaining labs (%))	Pass rate (%)	Accuracy % bias		Precision CV
			Mean % bias (Mean ± SD)	Mean absolute % bias (Mean ± SD)	Mean within-sample CV (Mean ± SD)
1	78	91.00%	−0.56 ± 1.31%	1.30 ± 0.72%	0.70 ± 0.34%
2	78 (100.0%)	92.30%	−0.24 ± 1.22%	1.14 ± 0.60%	0.69 ± 0.30%
3	47 (60.3%)	100.00%	0.00 ± 1.28%	1.20 ± 0.59%	0.63 ± 0.30%
4	36 (46.2%)	94.40%	−0.22 ± 1.25%	1.09 ± 0.69%	0.67 ± 0.34%
5	34 (43.6%)	91.20%	0.07 ± 1.34%	1.23 ± 0.60%	0.61 ± 0.27%
6	29 (37.2%)	100.00%	−0.11 ± 1.15%	1.04 ± 0.54%	0.64 ± 0.25%
7	26 (33.3%)	100.00%	−0.02 ± 1.27%	1.12 ± 0.65%	0.63 ± 0.27%
8	22 (28.2%)	95.50%	−0.28 ± 0.89%	0.90 ± 0.43%	0.67 ± 0.23%
9	18 (23.1%)	100.00%	−0.26 ± 1.05%	0.93 ± 0.59%	0.61 ± 0.35%
10	9 (11.5%)	100.00%	−0.29 ± 0.78%	0.66 ± 0.51%	0.68 ± 0.29%

Table 4. Performance of participants in HDL-cholesterol evaluation program

Event and p-value	Accuracy% bias		PrecisionCV
	Mean % bias(Mean ± SD)	Mean absolute % bias(Mean ± SD)	Among-run CV(Mean ± SD)
Initial	−1.86 ± 3.01%	4.22 ± 1.52%	1.56 ± 0.76%
Second	−0.06 ± 2.71%	2.88 ± 1.29%	1.58 ± 0.77%
p-value[a]	0.003	0.00002	0.88

[a]: p-value for comparison of initial and second events.

Table 5. Number of participants failing to meet specific CRMLN performance criteria for HDL-cholesterol

Event	Accuracy	Precision	Outliers	
	Absolute% bias± 5%	RunCV< = 4%	Within-method outliers	Between-method outliers
Initial	16	0	2	0
Second	2	1	3	0

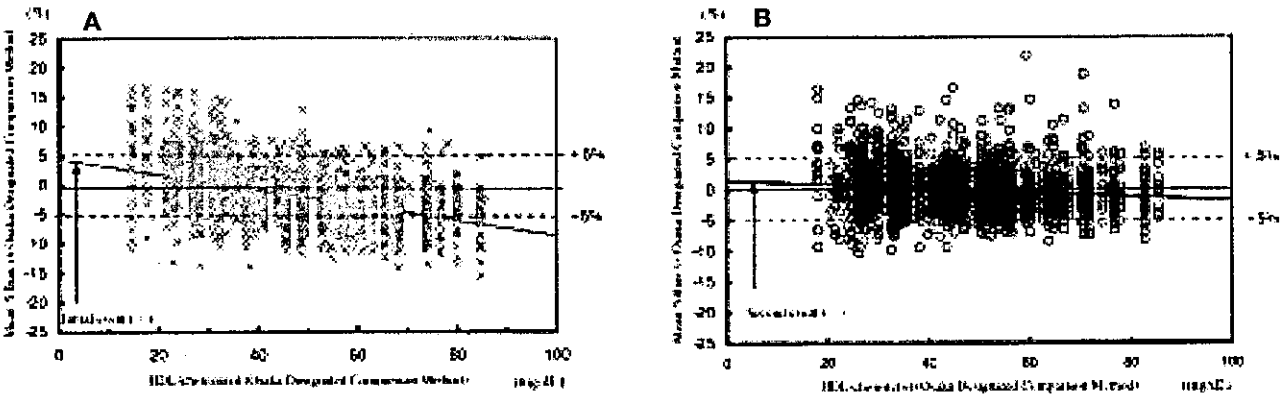

Fig. 2. Mean % Bias Plots for HDL-C. Bias plots of 46 Japanese clinical laboratories that participated in two evaluations of HDL-C performance. Mean percentage bias is plotted versus the HDL-C concentration (mg/dL) as determined by the designated comparison method at Osaka. Horizontal dotted lines mark the NCEP bias guidelines at ± 5%. Data for all participants is presented together in each plot. Figure 2 A: The regression line was $y = -0.1295x + 4.2998$ ($n = 46$, $r = -0.4549$) for the initial event(×). Figure 2 B: The regression line was $y = -0.0324x + 1.4207$ ($n = 46$, $r = -0.1380$) for the second event(○).

Table 5 summarizes the problems that some of these laboratories had in meeting the performance criteria. Precision was not a common problem. However, a number of laboratories had accuracy problems, particularly in the first event. The accuracy problems improved considerably in the second event of evaluations.

Discussion

Achieving accuracy within a laboratory and comparability between laboratories requires traceability to a defined common accuracy base. The most practical approach to achieve traceability is to ensure that manufacturers properly calibrate diagnostic products. This has been the primary focus of the CRMLN since its inception. Another approach to achieve comparability is to require that all clinical laboratories use the same method; however, this is neither practical or possible.

In Japan, no homogeneous systems exist where instrument, calibrators, and reagents are marketed by a single manufacturer. More commonly, heterogeneous systems are used where an instrument is purchased from one manufacturer, and calibrators and reagents are purchased from another manufacturer. A laboratory using a heterogeneous analytical system must assume primary responsibility for documenting performance and establishing traceability to the accuracy base. For this reason, many Japanese laboratories have chosen to participate in the CRMLN traceability programs. This offered us a unique opportunity to evaluate the impact of TC standardization and HDL-C evaluation by comparison of the initial and subsequent performances by clinical laboratories using all heterogeneous analytical systems.

Japanese manufacturers set accurate values on calibrators in three ways: 1) by performing a fresh sample comparison with OMC; this approach has been used for both TC and HDL-C; 2) by sending the calibrator to OMC for value-assignment; this approach has been used for HDL-C; and 3) OMC certifying enzymatic methods through the manufacturerís certification protocol; the manufacturers then use the enzymatic methods to assign calibrator values in-house.

For TC, accuracy by the certified laboratories tended to improvement in the second standardization compared to the initial standardization, however the improvement was not statistically significant. Although the mean bias for all laboratories was reduced by half between the initial and second performances, the difference is not statistically significant because of the relatively high variation between laboratories for this parameter. The *p*-value (0.11) observed for the *t*-test of the mean performance for the entire group of laboratories was not significant at the 95% level, but suggested a real difference. Although bias still exists, the smaller bias than in the initial standardization indicates that the laboratories improved their accuracy, namely the change in bias was in the desirable direction. The variability used to test this reflects among-laboratory variation that reduces the likelihood of finding a significant result.

The statistical tests to evaluate individual laboratoriesí performance between the first and second rounds confirm the suggestion of improvement in performance. This second approach avoids integrating the among-laboratory variability and is more powerful and confirms a trend for laboratories to improve between the first and second rounds.

The mean overall precision for this group of laboratories stayed very nearly the same between the initial and second performances. This is consistent with the fact that precision is not a problem with TC measurements. In fact, none of the laboratories failed to be certified because they did not meet the precision criterion.

When a laboratory failed to meet the criteria, OMC consulted with the laboratory to assist in determining the sources of and resolving the problem. The consultation would consist of a telephone call and/or a visit to OMC from laboratory personnel. If the source of the problem was determined to be with the calibrators or reagents, OMC consulted with the manufacturer to assist in resolving the problem. After the source of the problem was resolved, the laboratory had the opportunity to immediately participate in the certification protocol again. This consultation and certification procedure was followed until the laboratory could verify that it met the performance criteria.

Accuracy failure occurred in 11 laboratories during the first two rounds. The accuracy problem was resolved by changing the calibrator lot (two laboratories), changing of calibrator supply source (five laboratories), or stabilizing an unstable instrument (one laboratory). However, the cause in inaccuracy was not resolved in three laboratories.

The mean absolute percentage bias had a significant trend to lower values as laboratories continued participation in the certification program. We believe that this emphasizes the importance of regular participation with six months interval for TC over at least three years. We observed that, in general, laboratories that met the certification criteria in the first round continued to improve their bias the longer they participated in the program.

For HDL-C, accuracy was significantly improved in the second evaluation over the initial evaluation (34). Precision did not markedly improve. In the HDL-C evaluations, precision failure was resolved by maintenance of an unstable instrument. The accuracy problems that occurred in nine laboratories were resolved by reconstitution of the calibrator in five laboratories and a change of calibrator lot in three laboratories. The cause of inaccuracy was not resolved in one laboratory. Failure because of within-method outliers was resolved by readjustment of an unstable instrument in three laboratories and by a change in technologist in one laboratory. The cause of within-method outliers remained unresolved in one laboratory.

We understand that it will be desirable for using fresh-non-frozen samples for HDL-C measurement. However, in this study the fresh-frozen serum samples stocked at −60°C or below were used because first the HDL-C measurement should be divided among five analytical runs, namely five weeks as one run in a week and because second the reports are available for HDL-C can be determined accurately after storage at −70°C for up 1 month to 1 or 2 years (19,35). Any change in the samples was not found in the check of lipoprotein electrophoresis.

Commercial available kits for HDL-C measurement are developed based on various methodological principles (34). For this reasons the differences sometimes produce serious discrepancy among them for the patient samples that may have specific lipoprotein abnormality. This problem has not clearly been sorted and reported. Therefore, the manufacturers reagent kits should be furthermore focused to improvement for measurement of patient samples with lipid disorders.

In conclusion, accuracy for TC tended to improvement although not significantly, but for HDL-C improved between the initial and subsequent events. Precision was not significantly changed for either TC or HDL-C between the initial and subsequent rounds. Sustained participation in the TC certification program for 5 years demonstrated improved performance the longer a laboratory remained in the program, even while meeting the CRMLN performance criteria. We believe that continuous participation in the international standardization program from every clinical laboratory in Japan is a very essential part not only of the clinical or epidemiological study and practice for the risk management treatment but also of overseas publication of results in medical research involving Japanese peoples. The results of this study demonstrate that, at the outset of participation in the certification program, inaccuracy in TC and HDL-C testing was more of a problem than imprecision. CRMLN certification protocols will contribute effectively to improved accuracy for TC and HDL-C measurements.

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Papers

Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients

Antithrombotic Trialists' Collaboration

Abstract

Objective To determine the effects of antiplatelet therapy among patients at high risk of occlusive vascular events.

Design Collaborative meta-analyses (systematic overviews).

Inclusion criteria Randomised trials of an antiplatelet regimen versus control or of one antiplatelet regimen versus another in high risk patients (with acute or previous vascular disease or some other predisposing condition) from which results were available before September 1997. Trials had to use a method of randomisation that precluded prior knowledge of the next treatment to be allocated and comparisons had to be unconfounded—that is, have study groups that differed only in terms of antiplatelet regimen.

Studies reviewed 287 studies involving 135 000 patients in comparisons of antiplatelet therapy versus control and 77 000 in comparisons of different antiplatelet regimens.

Main outcome measure "Serious vascular event": non-fatal myocardial infarction, non-fatal stroke, or vascular death.

Results Overall, among these high risk patients, allocation to antiplatelet therapy reduced the combined outcome of any serious vascular event by about one quarter; non-fatal myocardial infarction was reduced by one third, non-fatal stroke by one quarter, and vascular mortality by one sixth (with no apparent adverse effect on other deaths). Absolute reductions in the risk of having a serious vascular event were 36 (SE 5) per 1000 treated for two years among patients with previous myocardial infarction; 38 (5) per 1000 patients treated for one month among patients with acute myocardial infarction; 36 (6) per 1000 treated for two years among those with previous stroke or transient ischaemic attack; 9 (3) per 1000 treated for three weeks among those with acute stroke; and 22 (3) per 1000 treated for two years among other high risk patients (with separately significant results for those with stable angina ($P = 0.0005$), peripheral arterial disease ($P = 0.004$), and atrial fibrillation ($P = 0.01$)). In each of these high risk categories, the absolute benefits substantially outweighed the absolute risks of major extracranial bleeding. Aspirin was the most widely studied antiplatelet drug, with doses of 75-150 mg daily at least as effective as higher daily doses. The effects of doses lower than 75 mg daily were less certain. Clopidogrel reduced serious vascular events by 10% (4%) compared with aspirin, which was similar to the 12% (7%) reduction observed with its analogue ticlopidine. Addition of dipyridamole to aspirin produced no significant further reduction in vascular events compared with aspirin alone. Among patients at high risk of immediate coronary occlusion, short term addition of an intravenous glycoprotein IIb/IIIa antagonist to aspirin prevented a further 20 (4) vascular events per 1000 ($P < 0.0001$) but caused 23 major (but rarely fatal) extracranial bleeds per 1000.

Conclusions Aspirin (or another oral antiplatelet drug) is protective in most types of patient at increased risk of occlusive vascular events, including those with an acute myocardial infarction or ischaemic stroke, unstable or stable angina, previous myocardial infarction, stroke or cerebral ischaemia, peripheral arterial disease, or atrial fibrillation. Low dose aspirin (75-150 mg daily) is an effective antiplatelet regimen for long term use, but in acute settings an initial loading dose of at least 150 mg aspirin may be required. Adding a second antiplatelet drug to aspirin may produce additional benefits in some clinical circumstances, but more research into this strategy is needed.

Editorial by FitzGerald

Correspondence to: Antithrombotic Trialists' Secretariat, Clinical Trial Service Unit, Radcliffe Infirmary, Oxford OX2 6HE
www.ctsu.ox.ac.uk

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Introduction

Previous meta-analyses of randomised trials have shown that antiplatelet therapy prevents serious vascular events,[1] arterial occlusion,[2] and venous thromboembolism[3] among a wide range of patients at high risk of occlusive vascular events. The proportional reduction in serious vascular events (non-fatal myocardial infarction, non-fatal stroke, or death from a vascular cause) was about one quarter in a wide range of high risk patients, irrespective of why the risk was high and irrespective of age, sex, blood pressure, or history of diabetes.[1]

The previous meta-analyses, however, left some important clinical questions unanswered. For instance, although long term antiplatelet therapy was shown to be of substantial benefit after ischaemic stroke, it was

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Details of the studies that were included, names of the collaborators, and a figure showing analyses of the proportional effects of treatment for different outcomes are available on bmj.com

not known whether antiplatelet drugs were of net benefit as an immediate treatment in the acute phase of such strokes.[4] There was also some uncertainty about whether antiplatelet therapy was of net benefit in patients with chronic conditions such as atrial fibrillation, stable angina, and atherosclerotic peripheral arterial disease that had been less extensively studied. Daily doses of at least 75 mg of aspirin had been shown to be effective in long term use, but theoretical advantages had been proposed for lower doses.[5]

The previous meta-analyses included only those trials that were available in 1990, and since then there have been many additional trials of aspirin at various doses and of other antiplatelet drugs.[6,7] There have also been trials of the effects of adding to aspirin another antiplatelet drug with a different mechanism of action. In addition, although certain anticoagulant regimens were known to be effective for particular high risk patients in the absence of antiplatelet therapy, it was not known whether the addition of anticoagulants to antiplatelets would provide additional protection. We have therefore updated previous meta-analyses to include studies available by September 1997. This paper summarises the updated results from the trials of antiplatelet drugs among high risk patients.

Methods

The methods and definitions used in the present meta-analysis were broadly similar to those used in the previous meta-analysis.[1]

Identification of trials

Details of each trial included in the analysis are available on bmj.com. The aim was to identify all trials, published or otherwise, that were available by September 1997 and that compared an antiplatelet regimen with a control or one antiplatelet regimen with another among patients considered to be at high annual risk (for example, over 3% a year) of vascular events because of evidence of pre-existing disease (previous occlusive event or predisposing condition). We included only those trials that were believed to have used a randomisation method that precluded prior knowledge of the next treatment to be allocated (thus, alternation or odd or even dates would not suffice) and were "unconfounded"—that is, contained two randomised groups that differed only with respect to the antiplatelet comparison of interest. Trials of oral antiplatelet regimens were eligible only if they had assessed more than one day of treatment, but we included trials of parenteral antiplatelet regimens of any duration. An antiplatelet drug was defined as one whose primary effect on the vascular system is to inhibit platelet adhesion, platelet aggregation, or both.[1]

We identified relevant trials by searching several electronic databases (Medline, Embase, Derwent, Scisearch, and Biosis; search strategy available on request); searching the trials registers of the Cochrane Stroke and Peripheral Vascular Disease Groups; manual searching of journals, abstracts, and proceedings of meetings; scrutinising the reference lists of trials and review articles; and inquiry among many colleagues, including representatives of pharmaceutical companies.

Definition of outcomes

The primary measure of outcome was a "serious vascular event" (that is, non-fatal myocardial infarction, non-fatal stroke, or death from a vascular cause and including any death from an unknown cause because most deaths in high risk patients are likely to be due to vascular causes). In order to allow the number of serious vascular events to be derived by adding the numbers of non-fatal myocardial infarctions, non-fatal strokes, and vascular deaths, we considered an event non-fatal only if the patient survived to the end of the scheduled follow up period (or died of a definitely non-vascular cause). Each contributing trialist's definition of a particular outcome (such as myocardial infarction) was used for counting vascular events, and we included all events classified by the trialist as probable or definite.

Deaths were divided into those with a vascular cause (defined as cardiac, cerebrovascular, venous thromboembolic, haemorrhagic, other vascular, or unknown cause) and those that were considered definitely non-vascular. Strokes were subdivided into intracranial haemorrhages (including intracerebral, subdural, subarachnoid, and extradural haemorrhages) and strokes of ischaemic or unknown aetiology; transient ischaemic attacks were not to be included. Major extracranial bleeds were those occurring outside the cranial cavity that were considered by the trialist to be serious (which, in general, meant that the patient required admission to hospital or blood transfusion). If during the trial a patient experienced more than one type of non-fatal outcome—for example, a myocardial infarction followed by a stroke—both events were recorded, but such patients contributed only once to the composite outcome of serious vascular event. If during the trial a patient experienced more than one non-fatal event of the same type (for example, two myocardial infarctions) or more than one pathological type of stroke (for example, a haemorrhagic stroke and an ischaemic stroke), only the first was to be recorded.

Data requested

We asked the coordinators of all potentially eligible trials for details about method of randomisation, blinding of treatment allocation, scheduled duration of treatment, and, if different, scheduled duration of follow up. Investigators for trials that had randomised at least 200 patients were asked to contribute, for each patient originally randomised, data on baseline characteristics (age, sex, blood pressure, and medical history) and dates of randomisation, follow up, and any vascular events that had occurred. In addition, we asked them for a tabular summary of the numbers of patients originally allocated to each treatment group (that is, without any post-randomisation exclusions) and the numbers of patients experiencing particular outcomes during the scheduled follow up period. These outcomes were non-fatal myocardial infarction, non-fatal stroke (haemorrhagic or other), non-fatal or fatal pulmonary embolism, death from a vascular or unknown cause, death from a definitely non-vascular cause, and major extracranial bleeding. Investigators responsible for trials that had randomised fewer than 200 patients were asked only for the tabular summary of the numbers of patients and outcomes (although a few such studies did contribute individual patient data).

In trials assessing a month or more of treatment, we intended that analyses would be of events occurring during the scheduled treatment period, but in two trials follow up data were available only for a period in excess of the scheduled treatment period (see bmj.com for details).[8 9] In trials with shorter courses of treatment, we analysed events during a period as close as possible to one month after randomisation. We checked data both for internal consistency and for consistency with relevant published reports and referred queries back to trial coordinators. Especially when data on individual patients were provided, the calculated numbers of vascular events may differ slightly from those reported in trial publications. Occasionally, when trial data had been discarded by investigators or were otherwise not available, the numbers of vascular events could be determined only from published reports.

Statistical methods

Proportional and absolute effects of treatment

We stratified analyses by trial to avoid direct comparisons between individuals in different studies. We calculated the observed minus the expected number of events, and its variance, from standard 2×2 tables of outcome by treatment. These were then summed over trials to give the grand total for observed minus expected events (O−E) and its variance (V). We then based significance tests on comparison of $z=(O-E)/\sqrt{V}$ with the standard normal distribution; P denotes the two sided significance level and $P>0.05$ is non-significant by convention. The typical odds ratio for these trials was calculated by the one step method[10] from $b=(O-E)/V$, either as exp(b) or, for rare events, as $(2+b)/(2-b)$. For odds ratios between 0.5 and 2 these two methods give almost identical answers.

Some trials used a deliberately unequal randomisation ratio and so had a substantial imbalance in the numbers of patients in treatment and control groups. We multiplied the control group in such trials by an appropriate integer[1] when calculating "adjusted" control totals (although not when making other calculations). When comparing the percentages affected in the treatment and in the adjusted control groups, we calculated the standard error (SE) of the difference (D) between these percentages as D/z.

Effects in specific categories of trials

We compared different trials or groups of trials using standard χ^2 tests for heterogeneity or, where appropriate, tests for trend between the observed effects on vascular events (with appropriate allowance made for multiple comparisons). But, even where there is significant heterogeneity, groups of patients in whom treatment is particularly advantageous or relatively ineffective can be difficult to identify reliably. Especially when small numbers of patients in a particular category have been studied, it is important that "lack of evidence of benefit" when that category is considered on its own is not misinterpreted as "evidence of lack of benefit"[11] As antiplatelet therapy reduces vascular events in a wide range of patients at high risk of occlusive vascular disease, the relevant question in any particular category is whether there is convincing evidence that there is no material benefit from treatment.[12 13]

Table 1 Major changes in availability of data between previous and current meta-analyses

	No of patients		No of vascular events	
	Previous	Current	Previous	Current
Antiplatelet therapy *v* control:				
Previous stroke/transient ischaemic attack	10 255	18 270	2062	3530
Acute stroke	29	40 821	5	3528
Stable angina	551	2 920	69	352
Atrial fibrillation	1 792	2 770	195	466
Peripheral arterial disease	4 939	9 214	486	605
Diabetes	1 200	4 961	55	820
Particular regimens:				
Aspirin <75 mg *v* control	357	3 655	45	670
Aspirin <75 mg *v* aspirin ≥75 mg	56	3 570	7	488
Clopidogrel *v* aspirin	0	19 185	0	2033
Aspirin + dipyridamole *v* aspirin	5 317	10 404	628	1262
Aspirin + glycoprotein IIb/IIIa antagonist *v* aspirin	0	24 802	0	2733

Description of trials

We identified 448 apparently randomised trials comparing an antiplatelet regimen with a control or one antiplatelet regimen with another among high risk patients. After review and, in cases of doubt, consultation with trial coordinators, 166 trials were excluded: 52 were not properly randomised, 24 were confounded, three had large numbers lost to follow up, 13 were abandoned before any outcome data were collected, 20 had a cross-over design, and 54 had not systematically recorded any of the relevant outcome events. In addition, since the focus of the present analyses was on patients at high risk of occlusive arterial disease, we excluded trials among patients with dementia or occluded retinal veins (even if they had been included in the 1994 meta-analysis[1]). Insufficient information was available from 19 eligible trials among 3427 patients.

Details of the remaining 197 randomised trials that compared antiplatelet therapy versus control (195 with data on vascular events) and the 90 that compared different antiplatelet regimens (89 with data on vascular events) are available on bmj.com. Information on individual patients was available for trials that collectively included 59% of the vascular events, and in these trials fewer than 2% of patients were lost to follow up. (Further details of excluded trials and missing data are available on request.)

Results

Effects on serious vascular events among high risk patients

Information about serious vascular events (non-fatal myocardial infarction, non-fatal stroke, or vascular death) was available from 195 trials of antiplatelet treatment versus control among a total of 135 640 patients at high risk of occlusive arterial disease (compared with 142 among 68 814 previously[1]). There was substantial additional information about patients with a history of stroke or transient ischaemic attack, those treated early after an acute stroke, and those with stable angina, atrial fibrillation, peripheral arterial disease, or diabetes (table 1).

Overall, 7705 (10.7%) serious vascular events were recorded among 71 912 high risk patients allocated antiplatelet therapy versus an adjusted total of 9502 (13.2%) among 72 139 allocated control ($P<0.0001$: fig 1). When we subdivided the trials into five main

Category of trial	No of trials with data	No (%) of vascular events: Allocated antiplatelet	Adjusted control	Observed-expected	Variance
Previous myocardial infarction	12	1345/9984 (13.5)	1708/10 022 (17.0)	-159.8	567.6
Acute myocardial infarction	15	1007/9658 (10.4)	1370/9644 (14.2)	-181.5	519.2
Previous stroke/transient ischaemic attack	21	2045/11 493 (17.8)	2464/11 527 (21.4)	-152.1	625.8
Acute stroke	7	1670/20 418 (8.2)	1858/20 403 (9.1)	-94.6	795.3
Other high risk	140	1638/20 359 (8.0)	2102/20 543 (10.2)	-222.3	737.0
Subtotal: all except acute stroke	188	6035/51 494 (11.7)	7644/51 736 (14.8)	-715.7	2449.6
All trials	**195**	**7705/71 912 (10.7)**	**9502/72 139 (13.2)**	**-810.3**	**3244.9**

Heterogeneity of odds reductions between:
5 categories of trial: χ^2=21.4, df=4; P=0.0003
Acute stroke *v* other: χ^2=18.0, df=1; P=0.00002

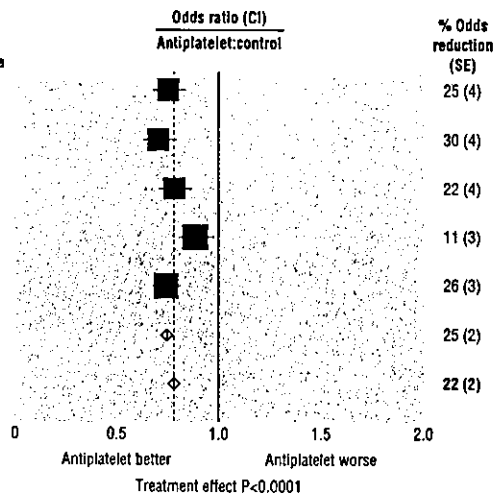

Fig 1 Proportional effects of antiplatelet therapy on vascular events (myocardial infarction, stroke, or vascular death) in five main high risk categories. Stratified ratio of odds of an event in treatment groups to that in control groups is plotted for each group of trials (black square) along with its 99% confidence interval (horizontal line). Meta-analysis of results for all trials (and 95% confidence interval) is represented by an open diamond. Adjusted control totals have been calculated after converting any unevenly randomised trials to even ones by counting control groups more than once, but other statistical calculations are based on actual numbers from individual trials

categories of high risk patient, there was clear evidence that the proportional reductions in serious vascular events differed among them (χ^2 for heterogeneity between these categories = 21.4, df = 4; P = 0.0003), mainly because of the somewhat smaller effect observed in patients treated during acute stroke (χ^2 for heterogeneity between acute stroke and other categories = 18.0, df = 1; P = 0.00002). Even so, the net benefit was highly significant both among patients with acute stroke (P = 0.0009) and, separately, among patients in each of the other high risk categories (each P < 0.0001).

Among patients with acute stroke, the absolute reduction in the risk of a serious vascular event was 9 (SE 3) per 1000 patients allocated antiplatelet therapy. This is smaller than the absolute benefit of 22 to 38 fewer vascular events per 1000 among the other four categories of high risk patients (fig 2). However, the net benefit in patients with acute stroke was achieved with less than one month of treatment, whereas the benefit among patients with a previous stroke or transient ischaemic attack (36 fewer events per 1000) resulted from an average of 29 months of treatment. Thus, the net benefit per month of antiplatelet treatment is substantially greater in the first month (starting at the time of the acute stroke) than it is during long term treatment for secondary prevention of stroke. Among patients with high risk conditions other than acute stroke, antiplatelet treatment produced a 25% (SE 2%) proportional reduction in serious vascular events that was similar in each of the four subcategories studied (χ^2 = 3.4, df = 3; NS, fig 1).

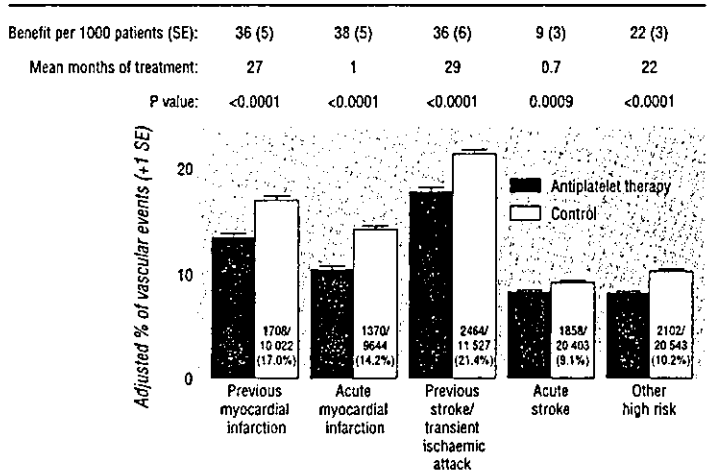

Fig 2 Absolute effects of antiplatelet therapy on vascular events (myocardial infarction, stroke, or vascular death) in five main high risk categories. Adjusted control totals have been calculated after converting any unevenly randomised trials to even ones by counting control groups more than once

Effects on different measures of outcome among high risk patients

Non-fatal myocardial infarction as outcome

Information was available on 2774 non-fatal myocardial infarctions after randomisation in 159 trials among high risk patients (compared with 2199 in 120 trials previously[1]) and on a further 4828 deaths attributed to coronary heart disease. Data for non-fatal myocardial infarctions are in figures 3a-3c (but had not been sought by the investigators in the main trials among acute stroke patients). Overall, antiplatelet treatment produced a 34% (3%) proportional reduction in non-fatal myocardial infarction (P < 0.0001; see figure on bmj.com) and a 26% (2%) reduction in non-fatal myocardial infarction or death from coronary heart disease (P < 0.0001). In each of the four categories of trial for which there was information, the reduction in non-fatal myocardial infarction was highly significant (each P < 0.001), although the proportional reduction seemed to be greatest in patients treated early after acute myocardial infarction (χ^2 for heterogeneity between subcategories = 12.3, df = 3; P = 0.006). But, even among patients who had not had an acute myocardial infarction, there was a clear reduc-

Table 2 Effects of antiplatelet therapy on fatal and non-fatal strokes, subdivided by stroke aetiology

Category of trial	No of fatal + No of non-fatal strokes/No of patients (combined %)		Stratified odds ratio (SE)	Adjusted absolute difference per 1000 (SE)
	Antiplatelet groups	Adjusted controls		
Probable or definite haemorrhagic stroke (in trials with at least one haemorrhagic stroke)				
Previous myocardial infarction	6+5/5476 (0.20)	6+8/5507 (0.25)	0.8 (0.4)	−0.5 (0.9)
Acute myocardial infarction	6+0/8821 (0.07)	1+1/8830 (0.02)	3.0 (1.4)	0.05 (0.03)
Previous stroke/transient ischaemic attack	35+26/9553 (0.64)	26+28/9610 (0.56)	1.2 (0.3)	0.8 (1.0)
Acute stroke*	68+140/20223 (1.03)	60+109/20205 (0.84)	1.2 (0.1)	1.9 (0.9)
Other high risk	22+9/4498 (0.69)	9+14/4529 (0.51)	1.3 (0.3)	1.8 (2.0)
Total	137+180/48571 (0.65)	102+160/48681 (0.54)	1.22 (0.10)	
			χ^2=2.5, df=4; NS	
Probable or definite ischaemic stroke (in trials with at least one haemorrhagic stroke)				
Previous myocardial infarction	17+40/5476 (1.04)	17+65/5507 (1.49)	0.69 (0.14)	−4.5 (2.1)
Acute myocardial infarction	8+29/8821 (0.43)	14+51/8830 (0.75)	0.58 (0.15)	−3.2 (1.1)
Previous stroke/transient ischaemic attack	106+662/9553 (8.04)	136+876/9610 (10.53)	0.75 (0.05)	−24.0 (5.1)
Acute stroke	90+231/20223 (1.59)	125+335/20205 (2.28)	0.69 (0.06)	−6.9 (1.4)
Other high risk	45+110/4498 (3.45)	69+166/4529 (5.19)	0.63 (0.09)	−17.4 (4.0)
Total	266+1072/48571 (2.75)	361+1493/48681 (3.88)	0.70 (0.03)	
			χ^2=3.3, df=4; NS	
Strokes of any aetiology (in trials recording data on non-fatal strokes)				
Previous myocardial infarction	33+83/9222 (1.26)	51+129/9250 (1.95)	0.64 (0.10)	−6.9 (1.9)
Acute myocardial infarction	13+32/9300 (0.48)	19+54/9291 (0.58)	0.62 (0.15)	−3.0 (1.2)
Previous stroke/transient ischaemic attack	288+957/11493 (10.83)	314+1248/11527 (13.55)	0.77 (0.04)	−27.2 (5.1)
Acute stroke	275+432/20238 (3.49)	293+522/20220 (4.03)	0.89 (0.05)	−5.4 (1.9)
Other high risk	131+257/16607 (2.34)	152+364/16733 (3.08)	0.73 (0.06)	−7.5 (1.7)
Total	740+1761/66860 (3.74)	829+2317/67021 (4.75)	0.78 (0.03)	
			χ^2=8.6, df=4; P=0.07	

*Includes haemorrhagic transformation of original infarct.

tion of about one third in non-fatal myocardial infarction (31% (4%); P<0.0001).

Stroke as outcome

Information was available on 3522 non-fatal strokes after randomisation in 158 trials among high risk patients (compared with 1496 in 122 trials previously[1]) and on a further 1424 fatal strokes. Antiplatelet therapy produced a 25% (3%) proportional reduction in non-fatal stroke (P<0.0001, see bmj.com and fig 3), with no significant heterogeneity between the proportional reductions in the five high risk categories of patient (χ^2=5.8, df=4; NS). Among those trials that recorded at least one haemorrhagic stroke, subdivision of all strokes (fatal or not) according to aetiology indicated that there was a proportional increase in fatal or non-fatal haemorrhagic stroke of 22% (95% confidence interval 3% to 44%; P<0.01) and a proportional decrease in fatal or non-fatal ischaemic stroke of 30% (24% to 35%; P<0.0001), with no significant heterogeneity between the proportional effects on each of these types of stroke in the five high risk categories studied (χ^2=2.5 and 3.3 respectively; both non-significant; table 2). But, although the proportional changes in the incidence of haemorrhagic and ischaemic stroke were about equal (and opposite) and although the absolute risks and benefits of antiplatelet therapy differed substantially from one category of patient to another, in each category the absolute risks were smaller than the benefits, so in each category of patient the overall risk of stroke (including strokes of unknown type) was reduced significantly (table 2).

Given these findings, the overall effect on total stroke may be estimated in any specific category of patient by considering the net absolute effects among such patients of the combination of an increase of about one quarter in the risk of haemorrhagic stroke and of a decrease of about one quarter in the risk of ischaemic stroke. So, for example, the proportional reduction in total stroke incidence was only around one half as large among patients with acute stroke as among patients in other high risk categories (χ^2=8.6, df=4; P=0.07; table 2) because in the month after an acute stroke about a quarter of the recurrent strokes in the control group were attributed to haemorrhage (or, particularly, to haemorrhagic transformation of the original infarct) whereas in other circumstances only about 6% were. Among all control patients (see totals in table 2), the case fatality rate is higher for haemorrhagic strokes (102 fatal, 160 not) than for ischaemic strokes (361 fatal, 1493 not). This may explain, at least in part, why the proportional effect of antiplatelet therapy on fatal strokes (16% (7%) reduction) seemed smaller, albeit non-significantly, than the effect on non-fatal strokes (28% (4%) reduction).

Vascular and non-vascular deaths

Information was available on 9605 deaths attributed to vascular (or unknown) causes in 193 trials among high risk patients (compared with 5253 in 141 trials previously[1]). Antiplatelet therapy produced a highly significant 15% (2%) proportional reduction in vascular deaths (P<0.0001; see bmj.com and fig 3), with no significant heterogeneity between the proportional reductions in each of the five high risk categories of patient (χ^2=7.8, df=4; NS).

A further 1414 deaths were attributed to non-vascular causes, but there was no excess of such deaths (785/71 656 (1.1%) antiplatelet *v* 872/71 876 (1.2%) adjusted control; odds ratio 0.92, 95% confidence interval 0.82 to 1.03; NS). Hence, antiplatelet therapy also produced a clear reduction of about one sixth in all cause mortality (P<0.0001; see bmj.com). If,

Table 3 Effects of antiplatelet therapy on fatal and non-fatal major extracranial bleeds

Category of trial	No of fatal + No of non-fatal major bleeds/No of patients (combined %)*		Stratified odds ratio (SE)	Adjusted absolute excess risk/1000 (SE)
	Antiplatelet groups	Adjusted controls†		
Previous myocardial infarction	1+2/672 (0.45)	1+2/668 (0.45)	—	—
Acute myocardial infarction	2+26/9134 (0.31)	3+20/9136 (0.25)	1.2 (0.3)	0 (1)
Previous stroke/transient ischaemic attack	15+65/8276 (0.97)	7+32/8289 (0.47)	2.0 (0.3)	5 (2)‡
Acute stroke	60+135/20 195 (0.97)	43+73/20 178 (0.57)	1.7 (0.1)	4 (1)§
Other high risk	17+212/8881 (2.58)	17+135/8897 (1.71)	1.5 (0.1)	9 (3)‡
Total	95+440/47 158 (1.13)	71+262/47 168 (0.71)	1.6 (0.1)¶	

*Only trials with systematic recording of all major extracranial bleeds (and that recorded at least one such bleed) are included.
†Percentage adjusted for unbalanced randomisation (see statistical methods).
‡P<0.001.
§P<0.0001.
¶χ^2 for heterogeneity=2.6, df=4; NS.

however, such antiplatelet therapy did have a protective or adverse effect on some specific cause of death (such as a particular type of cancer), the foregoing analysis of all non-vascular deaths might well be too insensitive to detect this. Site specific data on deaths from cancer were not available, so the suggestions that aspirin might prevent intestinal cancer[14] or cause renal cancer[15] could not be examined directly.

Pulmonary embolism

Only 32 trials planning to record symptomatic pulmonary embolism had recorded at least one non-fatal event, and among them antiplatelet therapy significantly reduced the risk of fatal or non-fatal pulmonary embolism (150/32 777 (0.46%) antiplatelet *v* 200/32 758 (0.61%) adjusted control; odds reduction 25% (10%); P<0.01). In both the treatment group and the control group, about half of those who had a pulmonary embolism survived to the end of the trial. Hence, the risk reduction was about one quarter in both cases (although with wide confidence intervals). This proportional reduction is somewhat smaller than that found in the 1994 meta-analysis of trials among surgical and high risk medical patients (47/4716 (1.0%) *v* 129/4730 (2.7%); odds reduction 64%, 95% confidence interval 50% to 73%; P<0.0001)[5] and in the subsequent pulmonary embolism prevention trial (55/8726 (0.6%) *v* 91/8718 (1.0%); odds reduction 43%, 18% to 60%; P=0.002) among patients having hip or knee surgery.[16]

Major extracranial bleeds

Information was available on 787 major extracranial bleeds in 60 trials recording at least one such bleed. These were generally defined as bleeds that were fatal or required transfusion; among them, 159 (20%) caused death. Little information was available on major extracranial bleeds from the trials of long term treatment after a myocardial infarction. Overall, the proportional increase in risk of a major extracranial bleed with antiplatelet therapy was about one half (odds ratio 1.6, 1.4 to 1.8), with no significant difference between the proportional increases observed in each of the five high risk categories of patient ($\chi^2=2.6$, df=4; NS; table 3). The proportional increase in fatal bleeds was not significantly different from that for non-fatal bleeds, although only the excess of non-fatal bleeds was significant. There were too few fatal and non-fatal bleeds in any particular category to estimate the absolute risks directly. However, a useful estimate of the excess risk of a major extracranial bleed may be obtained indirectly by applying the proportional increase of about one half to the absolute risk of bleeding in that category of patients.

Effects in different categories of high risk patients

Patients with history of myocardial infarction

Among 18 788 patients with a history of myocardial infarction in 12 trials (compared with 18 573 such patients in 11 trials previously[1]), allocation to a mean duration of 27 months of antiplatelet therapy resulted in 36 (SE 5) fewer serious vascular events per 1000 patients (fig 2). This benefit reflects large and highly significant reductions in non-fatal reinfarction (18 (3) fewer per 1000; P<0.0001: fig 3a) and vascular death (14 (4) fewer/1000; P=0.0006) as well as a smaller, but still significant, reduction in non-fatal-stroke (5 (1) fewer/1000; P=0.002). These benefits were substantially larger than the excess risk of major extracranial bleeding, which was estimated indirectly from table 3 (as described above) to be about three additional major extracranial bleeds per 1000 patients allocated antiplatelet therapy—that is, an excess of about 1 such bleed per 1000 patients per year.

Patients with acute myocardial infarction

Data were available on 19 288 patients with suspected acute myocardial infarction in 15 trials (compared with 18 773 such patients in nine trials previously[1]), nearly all of whom were in the ISIS-2 trial.[11] Allocation to a mean duration of one month of antiplatelet therapy resulted in 38 (5) fewer serious vascular events per 1000 treated patients (fig 2). This reflects large and highly significant reductions in non-fatal reinfarction (13 (2) fewer/1000; P<0.0001: fig 3b) and in vascular death (23 (4) fewer/1000; P<0.0001), together with a small but significant reduction in non-fatal stroke (2 (1) fewer/1000; P=0.02). The net benefit is substantially larger than the excess risk of major extracranial bleeding (for example, from arterial lines), which was estimated to be about 1-2 additional major extracranial bleeds per 1000 patients allocated antiplatelet therapy.

Patients with a history of stroke or transient ischaemic attack

The amount of information available on the effects of prolonged antiplatelet therapy among patients with a history of stroke or transient ischaemic attack has increased substantially since 1990 (table 1). This is mainly because of the second European stroke preven-