LDLコレステロール直接法の現在の課題

Current issues on LDL-cholesterol direct assay

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はじめに

低比重リポ蛋白(LDL)は，1947年にOncleyらが発見し，2年後にGofmanらが超遠心法で分離した．しかし，簡便にLDLコレステロール(LDL-C)を測定することは困難だった．1972年にFriedewaldらが発表したLDL-Cを求める計算式[1]は，その簡便さから現在も臨床で使われている．1990年代後半，我が国の試薬メーカーは，LDL-Cを直接測定できる試薬(直接法)を開発し世界を驚かせた．我が国でLDL-C直接法が普及したのは，動脈硬化性疾患予防ガイドライン2007年版に'高コレステロール血症の診断や管理目標値にLDL-Cを用いる'と明記されたことと，2008年から特定健康診査・特定保健指導(特定健診)の必須項目にLDL-Cが採用されたことが大きな要因である．

2010年にMillerらは，疾患群では直接法で測定したLDL-Cが，基準法であるβ-quantification法(BQ法)で測定したLDL-Cと乖離することが多いと報告した[2]．我が国でも同様の検討が行われ，その結果が最近公表された[3]．

本稿では，まずLDL-C直接法の原理と性能について述べ，LDL-C直接法の標準化に向けて解決すべき問題について概説する．

1 LDL-C直接法の原理と現状

LDL-C直接法の試薬は，我が国では現在12社から販売されている[3]．そのうちの8社は試薬の製造および販売を，残りの4社は他社の試薬を導入し自社ブランドとして販売している．各試薬の測定原理は，完全には公開されていないが，選択的消去法と選択的阻害法の2つのグループに分けることができる．選択的消去法では，第1試薬に含まれる界面活性剤(試薬により他の成分もあり)でLDL以外のリポ蛋白を可溶化し，そのコレステロールを消去する．第2試薬は，LDLを別の界面活性剤で可溶化しLDLのコレステロールを定量する．選択的阻害法では，第1試薬でLDL以外のリポ蛋白を界面活性剤とある種の化合物で保護し，第2試薬でLDLを別の界面活性剤で可溶化してコレステロールを定量する．12社のうちセロテック，フレイヤ，協和メデックスは，選択的阻害法あるいはそれに近い方法を採用している．現在，市場シェアの約4割ずつを，協和メデックスと積水メディカルが占めている．この2社に和光とデンカ生研の2社を加えた4社が，LDL-C直接法の市場シェアの約9割を占める．

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2 LDL-C直接法の性能評価

検査の性能は，精密度と正確度という2つの指標で表すことができる．精密度とは，同じ検体を繰り返し測定したときのバラツキを表す指標である．通常は，異なった日に測定した場合のバラツキ(日差変動)と，同一の日に測定した場合のバラツキ(日内変動)を評価する．バラツキの大きさは，繰り返し測定したときの平均値と標準偏差を用いて，CV(coefficient of variation: 標準偏差÷平均値×100, %)で表す．LDL-C直接法のCVは，精度管理がきちんとされている検査室では，日差・日内変動のどちらも1%未満のことが多い．

一方，正確度とは，測定値がどれほど真値に近いかを表す指標である．通常は，正確度の指標として真値と測定値の差(バイアス)を用いる．しかし，LDLにはいわゆる'純品'というものがなく，疾患によってLDL組成も変化する．すなわち，LDL-Cには絶対的な標準物質も，絶対的な基準分析法も存在しない．そこで，CDC (Centers for Disease Control and Prevention)によるLDL-Cの標準化では，BQ法によって値付けしたLDL-C値を基準値としている．BQ法では，まず超遠心法で比重1.006より軽いリポ蛋白を除去して下層部分(BF)を得る．次に，BF中のアポB含有リポ蛋白をヘパリン・マンガン法で沈殿させ，HDL分画を得る．BFとHDL分画中のコレステロール量を化学的測定法であるAbell-Kendall法で定量し，その差をLDL-Cとする[4,5]．その測定手順から，BQ法のLDL-C値にも誤差が含まれることは明らかである．

1) 米国での検討

Millerらは，LDL-C直接法の8つの試薬(製造・販売している7社と販売のみ行っている1社の試薬)について，その性能を調べた[2]．175例の新鮮血(非疾患群37例，疾患群138例)を採取し，LDL-Cを直接法とBQ法の2つの方法で測定して両者を比較した．error component analysisでは，非疾患群では8試薬中5試薬でNational Cholesterol Education Program (NCEP)のtotal error (TE)の基準を満たしたが，疾患群では1社も基準を満たさなかった．この検討の信頼性には，幾つか疑問がある．

解析に用いられたerror component analysisは，臨床的なデータ解析に適用した実績がほとんどない．この方法では，測定値のバラツキ(CV_t)が，日差変動(CV_b)，同時再現性(CV_e)，検体に特異的な効果(CV_d)の3つによって決まると仮定している(表1)．このうちCV_dは，通常のCV値とは異なる特殊な方法で計算されていて，異常検体が数本あると大きく値が変わる欠点がある．特に今回問題となっている中性脂肪高値の領域では，対象となる患者が少なく，前提条件である患者測定値の連続性仮定を満たさない可能性が高い．また，米国の検討の疾患群には，LDL-Cが極端に低い患者やLDLの組成が特殊な患者が2割弱含まれていた．これが，試薬の性能以上に解析結果を悪くさせた可能性を否定できない．また，採血から測定までの時間が24-48時間以内と長く，試薬間でのキャリーオーバー(別の試薬による測定用セルの汚染)もあった可能性がある．得られたLDL-Cの値とCV値から，TEを計算しているが，CV値としてCV_tを用いている(図1-c)．しかし，LDL-C直接法の試薬の認証試験では，TEを求めるときにはCV_b(日差再現性から求める)を使っている．このように，別の方法で求めたCV値で計算したTEを，NCEPが勧告している基準で判定してよいのか判断が難しい．

2) 我が国での検討

Millerらの検討結果を受けて，我が国で販売されている12のLDL-C直接法の試薬について同様の検討を行った[3]．その内訳は，自社で製造・販売している8社，試薬を他社から導入して販売のみを行っている4社(オーソー，シーメンス，ベックマン・コルターは，導入元を開示していない)である．Millerらの検討で問題となった特殊な脂質異常症，胆汁うっ滞性肝疾患，著明な高トリグリセライド血症(TG≧1,000 mg/dL)などは，最初から対象から除外した．最終的に解析した173例の内訳は，非疾患群が49例，疾患群が124例で，食後採血の検体はそれぞれ30.6%，46.0%であった．Millerらのデ

表1　日米の試験結果の比較(CV値と%バイアス)

		デンカ生研	和光	シスメックス	セロテック	フレイヤ(UMA)	協和メデックス	東洋紡	積水メディカル
非疾患群									
日差変動(CV_b)	米	1.3	0.6	2.3	2.9	2.2	0.7	—	1.2
	日	0.7	0.9	0.8	1.0	1.1	0.5	1.8	0.8
同時再現性(CV_e)	米	2.8	1.8	0.7	1.4	0.9	0.7	—	1.5
	日	0.5	0.6	0.5	0.6	0.5	0.4	1.1	0.5
検体特異的な効果(CV_d)	米	5.4	2.0	3.4	0.0	1.2	3.2	—	3.8
	日	4.5	3.8	5.1	5.9	4.1	3.2	7.0	5.1
総合CV値(CV_t)	米	6.2	2.8	4.2	3.2	2.6	3.3	—	4.2
	日	4.6	4.0	5.2	6.0	4.3	3.3	7.4	5.1
平均バイアス(%)	米	0.2	1.1	−6.0	−6.2	−0.1	−1.1	—	−0.7
	日	0.0	0.5	−0.3	1.8	0.7	0.6	−0.1	0.4
TEがNCEP基準の上限または	米	13.5	6.8	−13.3	−11.9	5.3	−7.5	—	−8.8
下限よりはずれている割合(%)	日	9.6	8.8	10.6	14.9	9.7	7.4	14.7	11.3
疾患群									
同時再現性(CV_e)	米	2.2	1.8	0.9	1.3	1.5	1.1	—	2.0
	日	0.6	0.5	0.6	0.7	0.7	0.5	0.9	0.6
検体特異的な効果(CV_d)	米	10.5	6.0	10.8	9.0	13.8	9.6	—	6.0
	日	4.7	5.3	9.5	10.0	7.0	5.8	5.9	4.8
総合CV値(CV_t)	米	10.8	6.3	11.1	9.5	14.1	9.7	—	6.4
	日	4.8	5.4	9.6	10.1	7.1	5.8	6.2	4.9
平均バイアス(%)	米	−1.5	4.1	−7.8	−11.8	−0.4	−0.8	—	−1.7
	日	0.7	1.4	−0.7	1.6	1.2	1.3	−0.6	1.1
TEがNCEP基準の上限または	米	22.3	18.2	−25.9	−26.6	31.9	20.4	—	−13.5
下限よりはずれている割合(%)	日	10.8	13.0	20.3	24.3	16.7	13.8	12.2	11.5

我が国で検討した12社のLDL-C直接法の試薬のうち，自社で試薬の製造を行っている8社についてデータを示した．なお，東洋紡の試薬は米国で検討されていないため，上段にデータがない．

CV_tは，3つのCV値(CV_b，CV_e，CV_d)の変量効果(random effects)を反映したもので，$(CV_b^2+CV_e^2+CV_d^2)^{1/2}$により求められる．

ータと直接比較できるように，統計解析は彼らと同一の手法を用いた．

予備検討では，検体の濃縮や試薬間のキャリーオーバーがないこと，検体の温度と運搬時間が守られていることをチェックした．その結果，我が国の検討では，米国のデータよりCV値が大幅に改善した試薬もあった(表1)．

非疾患群では，セロテックと東洋紡の試薬を除くと，%バイアスは0に収束しておりほぼ正確にLDL-Cが測定されていた(図1-a)．疾患群では，I型とIII型高脂血症を除くとBQ法と直接法のLDL-Cがほぼ一致している試薬がある一方，トリグリセライドが高くなると直接法のLDL-CがBQ法のそれより高くなる検体が増える試薬もあった(図1-a，b)．セロテックとオーソーの試薬は，トリグリセライド値にかかわらず，BQ法と乖離する検体が認められ，試薬の特異性に問題があると考えられた．また，BQ法と直接法の値が乖離する検体に食後採血のものはほとんどなく[3]，食事の影響は少ないと考えられた．前項で述べた理由で，TE(%)がNCEPの基準にもう少しで到達しそうな試薬を，性能が悪いと単純に判定することには慎重である必要がある．試薬性能の改善とともに，更に異常値検体を増やしたうえでの再検討が必要である．日常臨床上遭遇する多くの症例では，性能が問題となる領域から外れるため，スクリーニングツールとしての利用は問題ないと思われる．

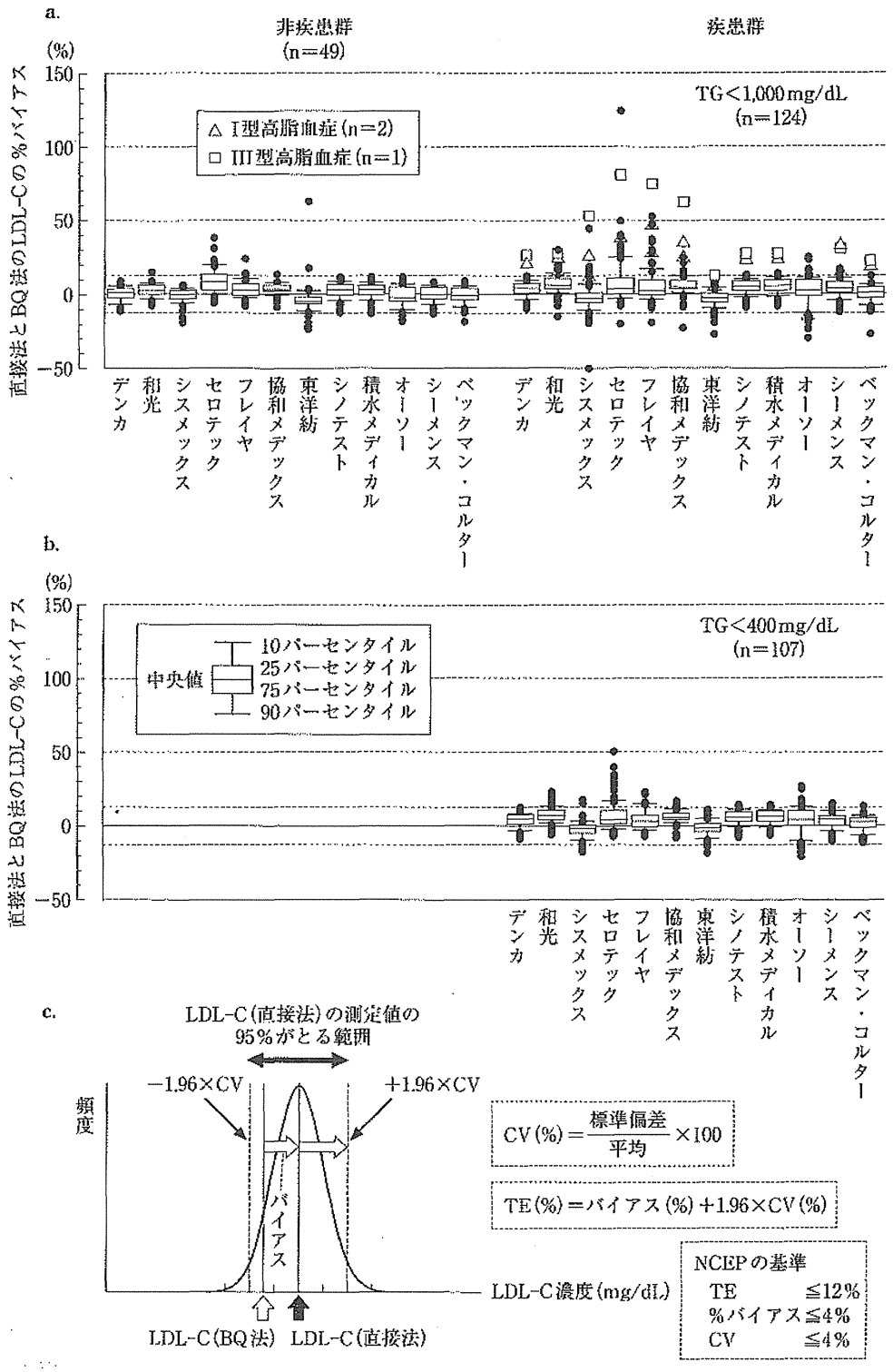

図1 直接法とBQ法のLDL-Cの%バイアス(文献[3]より改変)

$$\%バイアス=\frac{(直接法のLDL\text{-}C値)-(BQ法のLDL\text{-}C値)}{BQ法のLDL\text{-}C値}\times 100$$

a. 非疾患群と疾患群全体(トリグリセライド<1,000 mg/dL)

b. 疾患群(トリグリセライド<400 mg/dL)

c. CVとTE(total error)の計算法とその意味

TEは，直接法の測定値の95%が含まれる範囲の限界が，BQ法の値からどれほど離れているかを示す指標である．通常は，CV値は日差変動で求めたもの(表1のCV_b)を用いる．error component analysisでは，CV_Iを使用している(本文参照)．NCEPの基準は，TE≦12%，%バイアス≦4%，CV≦4%のすべてを満たすことを要求している．

3 LDL-C直接法の標準化に向けての課題

Friedewaldの式は，食後採血に適用できず，トリグリセライドが400 mg/dL未満であってもトリグリセライドが高くなるほど誤差が大きくなる[6]．LDL-C直接法の再現性の良さと食事の影響を考えなくともよい利点を生かすには，その標準化は必須である．脂質検査の標準化は，米国のCDCを中心としたCRMLN(Cholesterol Reference Method Laboratory Network)によって行われている．我が国では，循環器病研究センター(大阪府立健康科学センターより移転)にCDC/CRMLNの脂質基準分析室があり，試薬の認証試験を行っている．LDL-C直接法の試薬の反応性の違いは，高トリグリセライド血症の場合に明らかとなる傾向がある．しかし，現在のLDL-Cの標準化プログラムでは，認証試験のために高トリグリセライド血症の検体も集めるよう義務づけられていない．この点は，プロトコールの修正が必要だろう．

また，試薬間のLDL-C表示値の差を縮小させるために，キャリブレーターの値付けの方法を改善する必要がある．各メーカーのLDL-C値は，BQ法のLDL-Cの値とそれぞれ良好な相関を示すが，回帰直線の傾きは微妙に異なる．ほとんどのメーカーは，LDL-Cが120 mg/dL前後の検体を用いて，独自にBQ法でキャリブレーターを値付けしている．検査室では，メーカー指定のキャリブレーターを使ってワンポイントで検量線を引き，LDL-Cの測定値を算出している．したがって，値付けの微妙な違いが，メーカー間の測定値の系統的な差になる[7]．共通な検体を測定してキャリブレーターの値付けに反映させれば，測定値がもっと収束するのではないだろうか．

LDL-C直接法の標準化を更に進めるためには，検体を凍結しても測定値が変わらないような試薬の開発も望まれる．このような試薬を使えば，過去の保存検体を用いて，LDL-C直接法の臨床的意義を短期間に明らかにすることができる．

最後に，性能が不良であることが明らかとなった試薬は，販売を中止するか，改良が終わるまで販売を自粛すべきである．一部の試薬のために，LDL-C直接法への信頼性が揺らぎ，臨床で混乱を招いていることを自覚してほしい．

おわりに

LDL-C直接法の現状と問題点について述べた．LDL-C直接法の信頼性のあるチェック機構を構築するとともに，疫学調査や介入試験などにより直接法で測定したLDL-Cの意義を示すエビデンスを蓄積する必要がある．

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とを『*JAMA*』に世界ではじめて報告した[4]．ここで特記すべきことはFGF23で補正するともはや血清リン値は生命予後のリスク因子ではないことである．また，FGF23はCKDが軽度の段階(eGFR>30)に限ってはESRDを予測する因子であることも報告した．FGF23が全死亡を予測するのはCKDのどのステージであろうが同等であったが，このことと対照的であった．著者らもintact FGF23とビタミンDの栄養状態を示す25-hydroxyvitamin Dが腎予後[3]やCVD予後[5]を予測することを大阪のコホートで報告しており，これらの結果から人種を超えた再現性のある結果といえる．

FGF23がリンの負荷によって上昇することや，腎不全ではFGF23の共役因子である膜型klothoが低下している事実を考えると，FGF23が生命予後や腎予後を予測したことは非常に示唆に富む結果である．一般にCKD早期の患者にリンを数日にわたって経口的に負荷しても，かならずしも血清リン値は上昇をみない．この理由はFGF23やもうひとつのリン利尿因子であるPTHが代償的に上昇するからであり，CKD早期ではリン負荷量は血清リン値よりもこれらのホルモンにまず反映される．その意味でFGF23はリンの負荷量を反映するホルモンともいえる．また，リン負荷量とは独立して，腎機能が悪化するとFGF23が上昇するのは遠位尿細管におけるKlothoが低下し，これを代償するからかもしれない．いずれにせよ，FGF23は血清リン値よりもCKDの進行につれより早期に上昇するので，介入するべきターゲットになる可能性をはらんでいる．現在炭酸ランタンをはじめとするリン吸着薬がFGF23を下げることが報告されており，興味がつきない話題のたねとなっている．

CKDにおけるNgal

もう一点，CKD進行予知因子として面白い結果が出ているのはNgal(neutrophil gelatinase-associated lipocalin)である．Ngalはもともと急性腎障害(AKI)の尿細管マーカーであり，たとえば心血管手術後早期のNgalの上昇が術後のAKIを予測することで注目されていたが，CKDにおいてもNgalがeGFRの低下につれて上昇していることが報告されている．また，CKDにおいてNgalは尿蛋白量と相関が非常によく，従来の研究ではNgalが腎予後を予測するという報告はあるものの，不適切に尿蛋白が補正されていたという問題があった(あるいはまったく補正されていないなど)．CRICでは尿蛋白と腎予後との関連は線形ではないことが判明していたので，この点を考慮して尿蛋白を補正するとNgalは短期の腎予後(2年間のeGFR半減とESRD)は予測していたが，長期の腎予後は予測していなかった．この結果は近々論文化されるであろう．

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LDL-コレステロールの測定法

——直接法とFriedewald式

Methods for measuring LDL-cholesterol
——Direct method vs. Friedewald formula

LDL-コレステロール(LDL-C)は確立された冠動脈疾患の危険因子である．また，スタチンでLDL-Cを低下させると心血管系イベントは有意に減少する．このように，動脈硬化とLDL-Cの間には密接な関係がある．LDLは比重が1.006～1.063のリポ蛋白で，重量のおよそ半分をコレステロールが占める．LDL-Cは超遠心法でLDLを分離し，その分画中のコレステロールを定量して測定できる．しかし，超遠心法は時間と手間がかかりるうえ専用の機器も必要であり，日常検査には不向きである．

Friedewaldは1972年に総コレステロール，トリグリセリド(TG)，HDL-コレステロールの値からLDL-Cを推定する式(以下F式)を発表した[1]．F式は現在に至るまで広く臨床に用いられてい

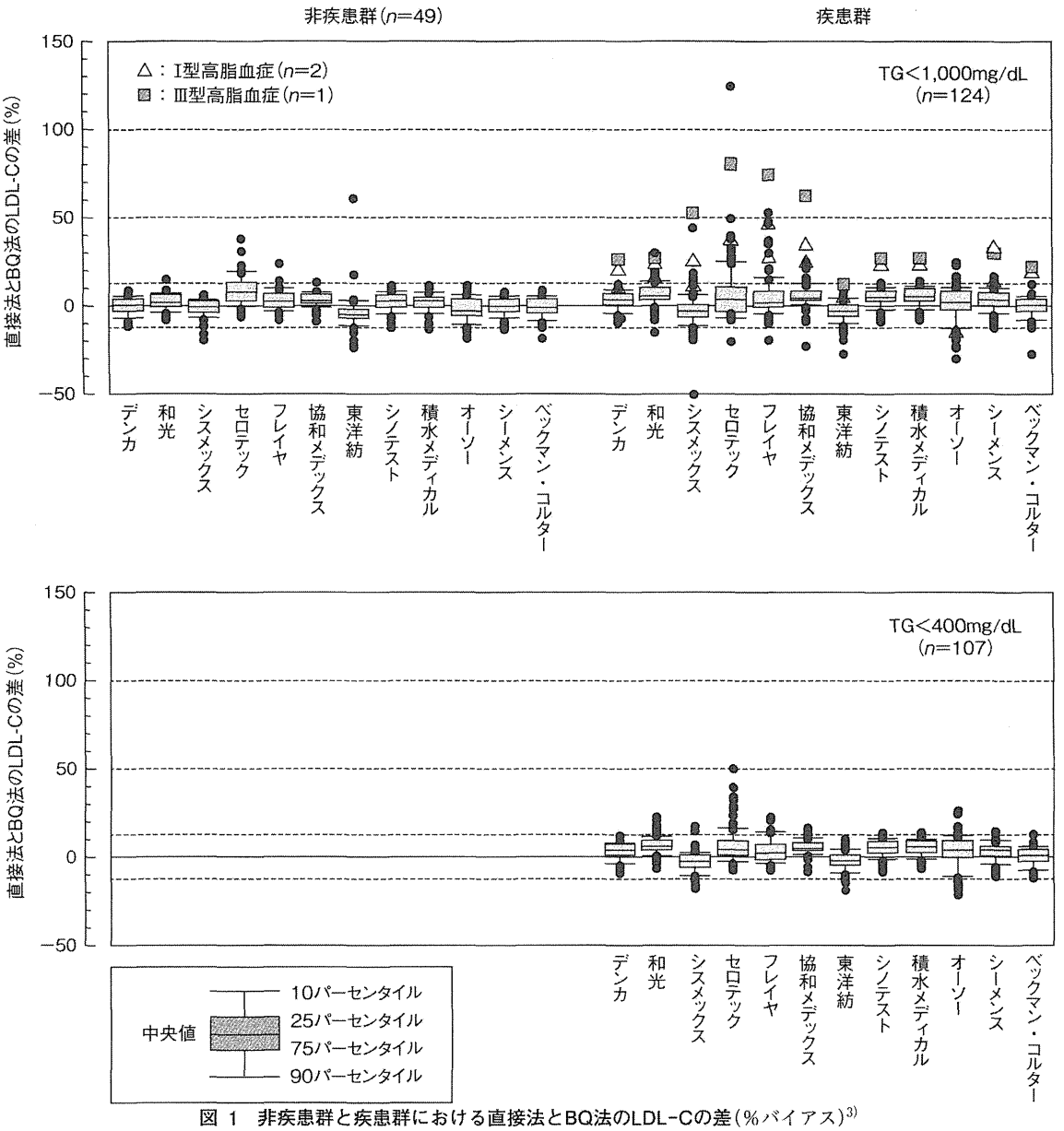

図 1　非疾患群と疾患群における直接法とBQ法のLDL-Cの差(%バイアス)[3]

直接法のLDL-C値からBQ法のLDL-C値を引いたもの(バイアス)を，BQ法のLDL-C値で割りパーセントで表示した．Millerらの報告より疾患群におけるバイアスのばらつきが著明に縮小している．

る．F式は空腹時に採血しTGが400 mg/dL未満という条件を満たす場合に適用できる．つまり医療機関の午後外来や健康診断などで，食後採血の場合には使えない．

LDL-C直接法の開発とMillerらの検討

1990年代後半，わが国の試薬メーカーが前処理を必要としないLDL-Cの測定法を開発した．これらはLDL-C直接法(以下，直接法)とよばれる．直接法は2008年にLDL-Cが特定健診の項目として採用されたのをきっかけとして，多くの施設に採用されるようになった．現在販売されている試薬はアメリカ疾病管理予防センター(CDC)が中心となって行っているLDL-Cの試薬の認証試験に合格したものである．2010年にMillerらは，直接法の試薬は非疾患群ではNational Cholesterol Education Program(NCEP)の基準を8試薬中5つが満たしたが，疾患群ではすべてが基準を満たさなかったと報告し大きな波紋をよ

んだ[2]．その後，彼らの報告の変動係数(再現性の指標)が通常より悪いこと，疾患群にLDL-Cの濃度や組成が正常とは極端に異なる検体が多かったことなど，複数の問題点がみつかった[3]．

わが国における LDL-C直接法の検討

そこで，わが国でも直接法の正確性を検討した[4]．わが国で製造販売されている12種類の直接法の試薬(うち4つは販売のみ)について直接法とCDCの基準法であるβ-quantification法(BQ法)でLDL-Cを同時に測定した．解析方法はMillerらと同じ方法を用いた．検体は非疾患群49例と疾患群124例から新鮮血を採取した．このうち，41.6%は食後検体であった．非疾患群ではセロテックと東洋紡の試薬を除けば，直接法のLDL-C値(LDL-$C_{直}$)はQB法のLDL-C値(LDL-C_{BQ})と同様の値を示した(図1)．一方，疾患群では両者の値が乖離する割合が非疾患群より高かった．また，測定値が乖離する場合の大部分で，LDL-$C_{直}$のほうがLDL-C_{BQ}より高値であった．この傾向はとくにⅠ型(n=2)およびⅢ型高脂血症(n=1)の患者で顕著であった．この3例を除くと，LDL-$C_{直}$とLDL-C_{BQ}が乖離する検体の少ない試薬グループと両者が乖離する検体がめだつ試薬グループに分けられた．セロテックとオーソーの試薬はTGが高い検体を除いてもLDL-$C_{直}$とLDL-C_{BQ}が乖離する検体が多かった．それ以外の試薬はTGが400 mg/dL未満の患者だけで検討するとLDL-$C_{直}$とLDL-C_{BQ}の値が乖離する検体は明らかに減少した．なお，2つの研究で使用された統計手法は通常用いられない特殊な方法である．解析する患者集団がすこし違うだけで，解析結果に影響がでる欠点がある．NCEPの基準値に近い値では結果の判定をより慎重に行う必要がある[4]．

おわりに

以上より，著者らの検討で正確性に問題があると指摘された試薬を除けば，直接法は再現性よく，食事の影響を受けずにLDL-Cを測定できる．しかし，TGが400 mg/dL以上の検体ではBQ法のLDL-Cと乖離が大きくなる試薬があることも事実である．直接法で測定したLDL-Cが本当に心血管イベント発症の危険因子であるのか，今後明らかにされるべきである．また，試薬の認証方法も高TG血症の検体を含めた臨床検体で行うなどの改善が望まれる．

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Validation of homogeneous assays for HDL-cholesterol using fresh samples from healthy and diseased subjects☆

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ABSTRACT

Background: High-density lipoprotein-cholesterol (HDL-C) is a negative risk factor for cardiovascular events. Although several homogeneous HDL-C assays are available, their accuracy has not been validated, particularly in subjects with disease. We aimed to clarify whether HDL-C concentrations measured by homogeneous assays [HDL-C (H)] agree with those determined by the reference measurement procedures [HDL-C (RMP)] using ultracentrifugation and precipitation with heparin-manganese reagent in fresh clinical samples.

Methods: HDL-C concentrations in samples from 48 healthy subjects and 119 subjects with disease were determined using 12 homogeneous assays and RMPs.

Results: All reagents showed excellent intra- and inter-assay CVs (<2.23%) for two pooled sera. Furthermore, the mean bias was within ±1.0% in nine reagents using samples from healthy subjects and in eight reagents using samples from subjects with disease. In a single HDL-C (H) determination, the total error requirement of the National Cholesterol Education Program (95% of results < 13%) was fulfilled in nine reagents using samples from healthy subjects and six reagents in those from subjects with disease. Error component analysis revealed that only one reagent exceeded ±10% total error in samples from healthy subjects, whereas four reagents exceeded this error in samples from subjects with disease. Correlations between HDL-C (H) and HDL-C (RMP) revealed that the slopes were within 1.00 ± 0.06 in six reagents in healthy subjects, and eight reagents in subjects with disease.

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Conclusions: Except for three reagents, HDL-C (H) agrees well with HDL-C (RMP) in subjects with common disease, but not in those with extremely low HDL-C or abnormal HDL composition.

1. Introduction

Lipoprotein profiles are closely associated with atherosclerotic disorders such as coronary artery disease and cerebrovascular disease, the major causes of death in industrialized countries [1,2]. Functionally, serum lipoproteins are divided into two groups: atherogenic and anti-atherogenic lipoproteins. Atherogenic lipoproteins consist of intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and remnant lipoproteins. Over a prolonged period of time, these lipoproteins accumulate in macrophages in the vascular walls; macrophages turn into foam cells, the main component of lipid-rich plaques. These are called "vulnerable plaques" because pro-inflammatory cells—including macrophages—degrade the fibrous cap of plaques using proteolytic enzymes, causing sudden rupture [3].

High-density lipoprotein (HDL) is the only circulating anti-atherogenic lipoprotein. HDL removes free cholesterol from somatic cells by accepting cell cholesterol via an exchange with the help of ATP-binding cassette transporter A1 (ABCA1) [4] and G1 (ABCG1) [5]. This reverse cholesterol transport pathway regresses vascular atheromatous plaques [6]. In clinical practice, the amount of HDL is expressed as the cholesterol concentration (HDL-C). Numerous cross-sectional and longitudinal epidemiological studies have shown that HDL-C is a strong negative risk factor for cardiovascular events [7,8]. In both primary and secondary prevention studies using statins, each 0.0259 mmol/L (1 mg/dL) increase in HDL-C level decreased the risk of coronary artery disease by 2–3% [9,10]. Since precipitation methods for HDL-C measurement are cumbersome and time consuming, HDL-C is measured by homogenous assays (so-called "direct assays") using a variety of principles in almost all clinical laboratories [11]. In general, homogeneous assays eliminate or inhibit non-HDL-C with the first reagents, and then solubilize HDL particles for measuring cholesterol with the second reagents. Although the performance of seven HDL-C homogeneous assays was investigated recently, the study subjects included a considerable number of patients with rare dyslipidemia and extremely low HDL-C [12]. Furthermore, there are additional novel assays based on different principles.

We examined the precision and accuracy of the HDL-C homogeneous assays that are commercially available at present. Using fresh blood samples from healthy subjects and subjects with disease, we compared the HDL-C concentrations determined using 12 homogeneous assays [HDL-C (H)] with those determined by the reference measurement procedures [HDL-C (RMP)].

2. Methods

2.1. Study subjects

This study was planned and carried out concurrently with the multi-center study of the precision and accuracy of homogeneous assays for LDL-cholesterol (LDL-C) published previously [13]. Initially, 173 subjects consisting of volunteers and patients with disease were recruited at the participating institutions. We excluded dyslipidemic patients with extremely low or high lipoprotein concentrations [triglyceride (TG) > 11.29 mmol/L (1000 mg/dL), LDL-C < 0.52 mmol/L (20 mg/dL), HLD-C < 0.52 mmol/L (20 mg/dL), and HDL-C > 2.59 mmol/L (100 mg/dL). In addition, we excluded patients with severe systemic infections, decompensated liver cirrhosis or cholestatic liver disease [13]. Healthy subjects ($n = 48$) were defined as normolipidemic healthy volunteers who had no abnormal laboratory tests or documented diseases. The remaining 119 persons were classified as subjects with disease, according to their medical history and lipoprotein profiles. At recruitment, written informed consent was obtained from all subjects.

The study protocol was reviewed and approved by the ethics committees of all participating institutions. This study was conducted according to the latest version of the Declaration of Helsinki.

2.2. Blood sampling and delivery

Fresh venous blood was drawn into vacuum tubes (Venoject II, VP-AS109K50, Terumo, Tokyo, Japan) from each subject regardless of time lapsed since the last meal. After the blood was allowed to clot, the serum was separated within an hour and poured into 50-mL plastic tubes (430290, Corning Japan, Tokyo) to equalize the blood components. Aliquots of samples were dispensed into screw-capped tubes and delivered to either SRL (Hachioji, Japan) for homogenous assays or Osaka Medical Center for Health Science and Promotion (OMC-HSP) (Osaka, Japan) for RMP [12]. Samples were placed in a cooling box containing refrigerant, and carried in a van equipped with a refrigerator. Temperatures were monitored continuously at two sites inside the box; temperatures were maintained between 2 and 4 °C within 24 h (Supplemental Fig. 1S).

2.3. HDL-C measurement

Within 24 h after blood collection, we measured HDL-C concentrations using 12 homogeneous assays, as well as RMP—described below. At SRL, the precision and accuracy of 12 homogeneous assays were evaluated (Reagent-A, Denka Seiken; Reagent-B, Wako; Reagent-C, Sysmex; Reagent-D, Serotec; Reagent-E, Fureiya; Reagent-F, Kyowa Medex; Reagent-G, Toyobo; Reagent-H, Shino-Test; Reagent-I, Sekisui Medical; Reagent-J, Ortho Clinical Diagnostics; Reagent-K, Siemens Healthcare, and Reagent-L, Beckman Coulter). Reagents-A–G, and Reagent-I were original homogenous assays, whereas Regent-H, Reagent-J, Reagent-K and Reagent-L were introduced products from other manufacturers (Supplemental Table S1). Reagents-A to -I were run on the same automated analyzer (Hitachi-917, also called Hitachi-7170 in Japan) that was used in the preceding studies [12,13]. The other three reagents were run on three different instruments manufactured by the distributors that produced the individual reagents. All of the reagents, calibrators and controls were supplied by the respective manufacturers and distributors. Under conditions of anonymity, the operators of SRL measured HDL-C (H) in triplicate as described previously [13]. In a preliminary study, we confirmed no cross contamination between cells, and no condensation during measurements using the Hitachi-910 instrument.

HDL-C was measured by RMP at OMC-HSP. First, two tubes prepared from each sample were ultracentrifuged at 18 °C, 105,000× g for 18.5 h. The bottom fraction was recovered by discarding the floating fraction using a tube slicer. After adding heparin-manganese solution to the bottom fraction, we removed the precipitate by centrifugation (1500× g for 30 min), and obtained the HDL fraction [14]. Finally, we measured cholesterol levels

of the supernatant in duplicate as HDL-C (RMP) by the Abell–Kendall method [15].

2.4. Statistical analysis

We determined three CVs (%): CV_b, derived from among-run variation using pooled serum; CV_e, derived from within-run variation using triplicate measurements; and CV_d, derived from patient-specific errors, as described previously. CV_t was calculated from these three CVs [12,13]. Bias was calculated by subtracting HDL-C (RMP) from HDL-C (H). Percentage total error (%TE) was calculated as the sum of %bias and CV_t multiplied by 1.96. We used the criteria of the National Cholesterol Educational Program (NCEP) for TE requirement for HDL-C measurement, where %bias, CV and % TE were less than 5, 4 and 13%, respectively [16].

3. Results

3.1. Subjects' characteristics

A total of 173 fresh samples were collected during the study period. Six subjects were diagnosed with hyper-α-lipoproteinemia with HDL-C > 2.59 mmol/L, and excluded from the analysis. Of six patients, two were diagnosed as heterozygotes for cholesteryl ester transfer protein (CETP) deficiency. Neither CETP activities nor CETP gene mutations were determined in the other patients. In all subjects, HDL-C ranged from 0.74 to 2.54 mmol/L, and TG ranged from 0.35 to 10.57 mmol/L (Table S2).

3.2. Homogeneous assay precision

In most reagents, the inter- and intra-assay CV values were less than 1.0% for pooled sera with HDL-C concentrations of 1.29 and 2.13 mmol/L (Table S3). The total CV values were 0.84–2.23%; this maximum value was about half of the target (4%) proposed by NCEP.

3.3. Relationship between HDL-C (H) and HDL-C (RMP)

In the healthy group, the %bias values of most samples were within 13% for all reagents, except Reagent-D. In contrast, a significant number of samples in subjects with disease exceeded 20% for Reagents-G and -L (Fig. 1). The medians deviated markedly from zero for Reagents-D and -G.

Scatter plots, % bias plots and Bland–Altman plots clearly showed that Reagents-G and -L had poorer analytical performance than the other reagents, particularly in the samples from subjects with disease. In most reagents, HDL-C (H) exhibited good correlation with HDL-C (RMP) in samples from healthy and diseased subjects (Fig. 2, upper panels). However, the intercepts and slopes of the linear regression lines ranged from −0.059 to 0.210 mmol/L and from 0.90 to 1.12 in the samples from healthy subjects, and from −0.016 to 0.316 mmol/L and from 0.89 to 1.01 in samples from subjects with disease (Table 1). In Bland–Altman plots, the absolute bias positively correlated with the mean value of HDL-C (H) and HDL-C (RMP) concentrations for Reagent-D (Fig. 3). There was marked diversity in the absolute bias independent of the mean HDL-C values for Reagents-G and -L.

3.4. Total error for single measurements

In samples from healthy subjects, five reagents fulfilled the requirement of NCEP in all samples, while Reagent-D failed in almost 40% of samples (Table 2-A). In samples from subjects with disease, nine reagents reached 90% agreement between HDL-C (H) and HDL-C (RMP). These reagents showed good agreement in even samples from patients with severe hypertriglyceridemia or type III hyperlipidemia (Table S4). In contrast, the percentages of agreement in Reagents-D, -G, and -L were markedly lower.

3.5. Error component analysis

This analysis was less informative than those using scatter plots and Bland–Altman plots. In samples from healthy subjects, all reagents met the NCEP requirement (Table 2-B). In samples from

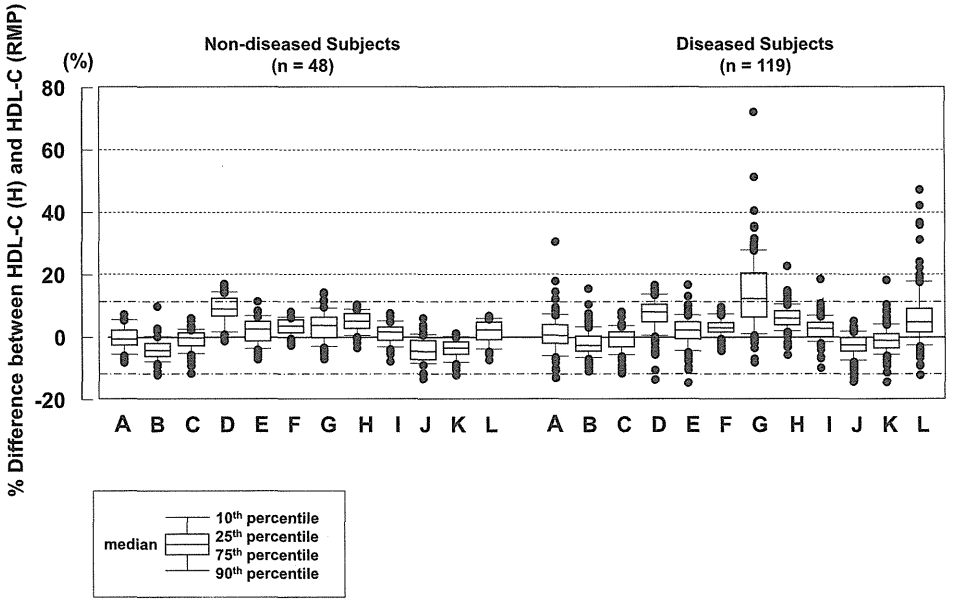

Fig. 1. Box-and-whisker plots of the percentage difference between HDL-C (H) and HDL-C (RMP) in samples from healthy and diseased subjects. HDL-C concentrations in fresh serum samples were measured using 12 homogeneous assays and RMPs. Percentage differences were determined using the first measurements of individual reagents.

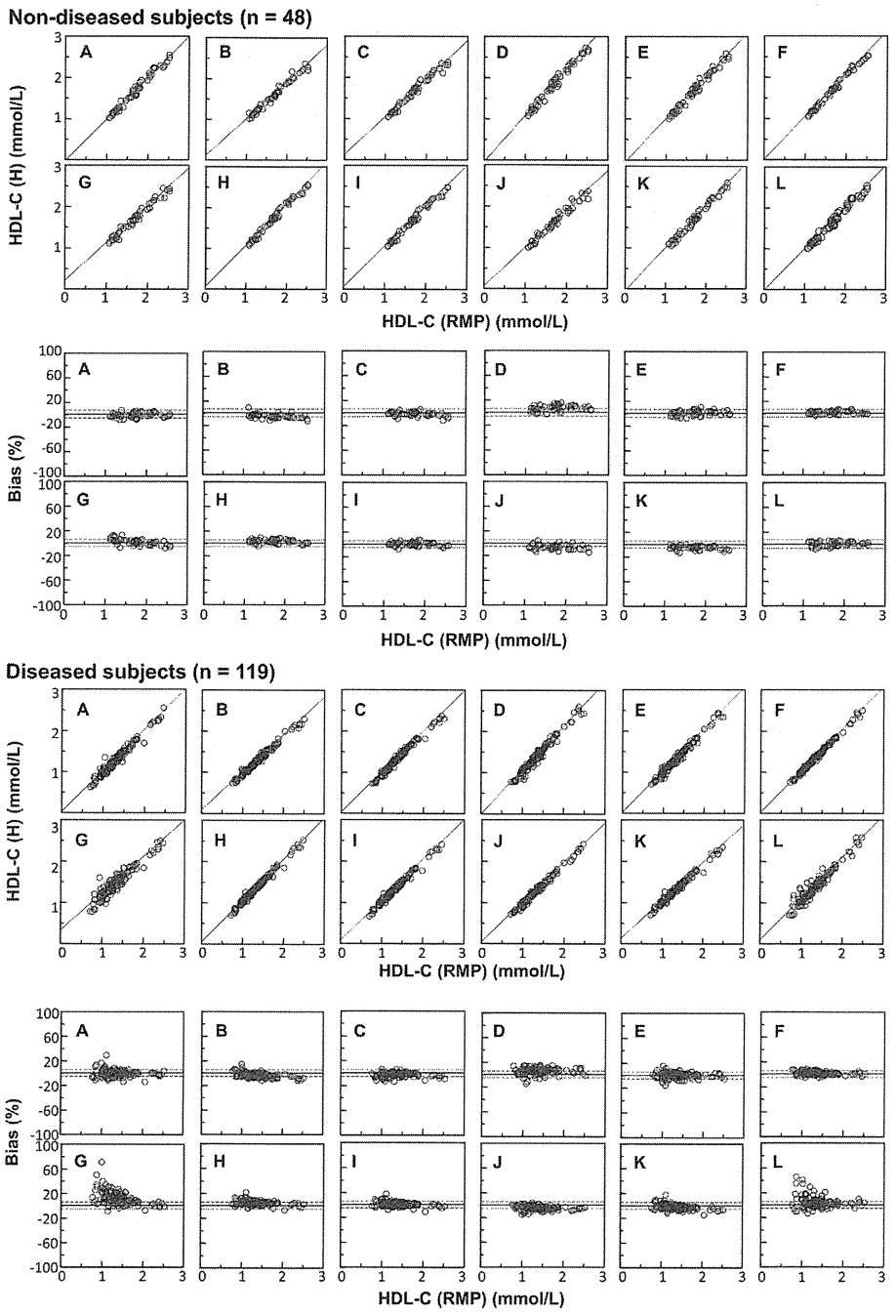

Fig. 2. Relationship between HDL-C (H) and HDL-C (RMP) in samples from healthy and diseased subjects. Data from healthy and diseased subjects were plotted as scatter graphs (upper panels) and %bias graphs (lower panels).

subjects with disease, Reagents-G and -L did not meet the NCEP requirement. The CV_d value was the critical determinant of these unfavorable results.

4. Discussion

Our data indicates that the HDL-C (H) concentrations determined by most of the homogeneous assay reagents agree well with HDL-C (RMP) determined by CDC reference method procedures in samples from both healthy and diseased subjects. Nine of the twelve reagents achieved better than 90% agreement with the NCEP total error requirement for a single HDL-C determination (Table 1). In contrast, the HDL-C (H) measured using Reagents-G and -L did not match the HDL-C (RMP) data over a wide range of HDL-C concentrations (Fig. 2).

Standardization of homogenous HDL-C assays is problematic because no pure and stable HDL particles are available for use as a reference. Apolipoprotein A-I is a better predictor for atherosclerotic disorders than HDL-C, and is already standardized with the reference material [17]. However, apolipoprotein A-I is not measured as widely as HDL-C, partly due to the relatively high cost. In 1994, the Cholesterol Reference Method Laboratory Network (CRMLN) launched a HDL-C certification program for manufacturers [18] using the designated comparison method (DCM) and

Table 1
Relationships between HDL-C (H) and HDL-C (RMP) values.

Subjects	Reagent											
	A	B	C	D	E	F	G	H	I	J	K	L
Non-diseased subjects (n = 48)												
Sy/x	2.60	2.20	2.42	2.83	2.70	1.75	2.56	2.16	2.19	2.74	2.04	2.24
Intercept (mmol/L)	−0.041	0.108	0.107	−0.052	−0.059	0.005	0.210	0.060	0.050	0.058	0.073	−0.038
95% C.I. (mmol/L)	−0.107 to 0.030	0.025 to 0.191	0.020 to 0.194	−0.137 to 0.034	−0.140 to 0.022	−0.048 to 0.059	0.121 to 0.299	−0.006 to 0.127	−0.016 to 0.116	−0.041 to 0.156	0.004 to 0.141	−0.107 to 0.032
p-value	N.S.	0.0122	0.0167	N.S.	N.S.	N.S.	<0.0001	N.S.	N.S.	N.S.	0.0375	N.S.
Slope	1.02	0.90	0.93	1.12	1.06	1.03	0.91	1.01	0.98	0.92	0.92	1.04
95% C.I.	0.98–1.07	0.85–0.95	0.87–0.98	1.07–1.17	1.01–1.11	1.00–1.07	0.85–0.96	0.97–1.05	0.94–1.02	0.86–0.99	0.88–0.96	1.00–1.08
p-value	N.S.	0.0002	0.0109	<0.0001	0.0205	N.S.	0.0007	N.S.	N.S.	0.0158	0.0003	0.0367
Diseased subjects (n = 119)												
Sy/x	2.60	1.79	1.96	2.72	2.31	1.33	4.44	1.85	1.85	1.70	1.93	3.96
Intercept (mmol/L)	0.005	0.114	0.025	−0.016	0.009	0.038	0.316	0.081	0.076	0.033	0.113	0.082
95% C.I. (mmol/L)	−0.024 to 0.034	0.077 to 0.152	−0.019 to 0.069	−0.078 to 0.046	−0.042 to 0.059	0.008 to 0.067	0.231 to 0.401	0.041 to 0.121	0.038 to 0.109	0.003 to 0.063	0.073 to 0.154	−0.003 to 0.167
p-value	N.S.	<0.0001	N.S.	N.S.	N.S.	0.0140	<0.0001	0.0001	0.0002	<0.0318	<0.0001	N.S.
Slope	0.99	0.89	0.98	1.09	1.01	1.00	0.89	1.00	0.97	0.95	0.91	1.00
95% C.I.	0.97–1.01	0.87–0.92	0.94–1.01	1.04–1.13	0.98–1.05	0.98–1.03	0.84–0.95	0.97–1.03	0.94–1.00	0.93–0.97	0.88–0.93	0.94–1.06
p-value	N.S.	<0.0001	N.S.	0.0003.	N.S.	N.S.	0.0002	N.S.	0.0305	<0.0001	<0.0001	N.S.

A, Denka Seiken; B, Wako; C, Sysmex; D, Serotec; E, Fureiya; F, Kyowa Medex; G, Toyobo; H, Shino-Test; I, Sekisui Medical; J, Ortho Clinical Diagnostics; K, Siemens Healthcare Diagnostics; L, Beckman Coulter.
Sy/x, standard error of the estimate; C. I., confidence interval.

Centers for Disease Control and Prevention (CDC) reference method (CDCRM). Precipitation methods, such as DCM, cannot completely precipitate apolipoprotein-B-containing lipoproteins in hypertriglyceridemic samples [19]; therefore, we measured HDL-C (RMP) at OMC-HSP by CDCRM, which included removal of chylomicron and very-low density lipoprotein (VLDL) by slicing a tube after ultracentrifugation, and precipitation of LDL from the bottom fraction with heparin-manganese solution. Unlike some reagents for LDL-C homogeneous assays, high TG concentrations were not associated with discrepancies between HDL-C (H) and HDL-C (RMP) (Table S4, S5). In addition, the prevalence of postprandial samples did not increase in the discordant results from diseased subjects (Table S5). These results suggest that it is acceptable to use postprandial HDL-C (H) data for calculation of the non-HDL-C concentration.

Miller et al. conducted a similar study of homogenous assays for HDL-C where they also examined the accuracy of homogeneous assays for LDL-C [12]. In subjects with disease, our results of error component analysis were better than those of Miller's study, although data were comparable in healthy subjects. We excluded patients who might have abnormal HDL particles due to genetic dyslipidemia, severe systemic infection, and decompensated liver cirrhosis. Furthermore, we also excluded samples with severe hypertriglyceridemia (TG > 11.29 mmol/L) since chylomicrons float spontaneously on the top of samples, which may cause mechanical problems with the sampling probes. Error component analysis is not applicable to samples in which the mean successive difference between HDL-C (H) and HDL-C (RMP) is large or discontinuous [13]. Miller et al. evaluated samples from 138 subjects with disease, including those with lecithin-cholesterol acyltransferase (LCAT) deficiency (n = 1), Niemann–Pick disease type B (n = 1), and primary biliary cirrhosis (n = 6) [12]. In LCAT deficiency, apoA-I containing HDL decreased markedly, while apoE-containing HDL was comparatively preserved [20]. In Niemann-Pick type B, the sphingomyelin content of HDL particles was greater than that in healthy subjects [21]. In cholestatic liver diseases such as primary biliary cirrhosis, apoE-rich HDL increases markedly [22,23]. In the present study, we excluded samples from subjects with hyper-α-cholesterolemia (HDL-C > 100 mg/dL) and the abovementioned diseases. We reported previously that the mean apoE-rich HDL-cholesterol concentration was more than fourfold greater in hyper-α-cholesterolemia than in healthy controls [24]. Due to the difference in the measuring principles, reactivity to apoE-rich HDL may vary markedly among the HDL-C homogeneous assay reagents. Sugiuchi et al. measured HDL-C in PBC and cholesteryl ester transfer protein (CETP) deficiency using six homogeneous assays. HDL-C (H) ranged from 51 to 147 mg/dL in PBC, and from 157 to 192 mg/dL in CETP deficiency [23]. Miller's study is likely to have overemphasized the inaccuracy of homogenous assays since they included a relatively high percentage of samples with extremely low HDL-C or extremely abnormal HDL composition. However, it should be noted that our data certify the analytical performance of homogenous assays in samples only from subjects with common diseases.

No significant problems were identified in the error component analysis of most of the HDL-C homogeneous assay reagents (Table 2-B). Thus, HDL-C homogeneous assays showed generally acceptable accuracy in the healthy and diseased groups, except for those from a few commercial sources. However, we suggest the need for further improvement with respect to standardization when examining the data in a serial manner. The scatter plots showing the relationships between HDL-C (H) and HDL-C (RMP) revealed that the slopes and Y-intercepts of the regression lines did not fall within the satisfactory range (1.00 ± 0.03 for slope, and 0.00 ± 0.06 for intercept; Fig. 2, Table 1). The slopes ranged from 0.90 to 1.12 in the healthy group, and from 0.89 to 1.09 in the diseased group. Even after excluding Reagents-D, -G and -L, three other reagents had significantly

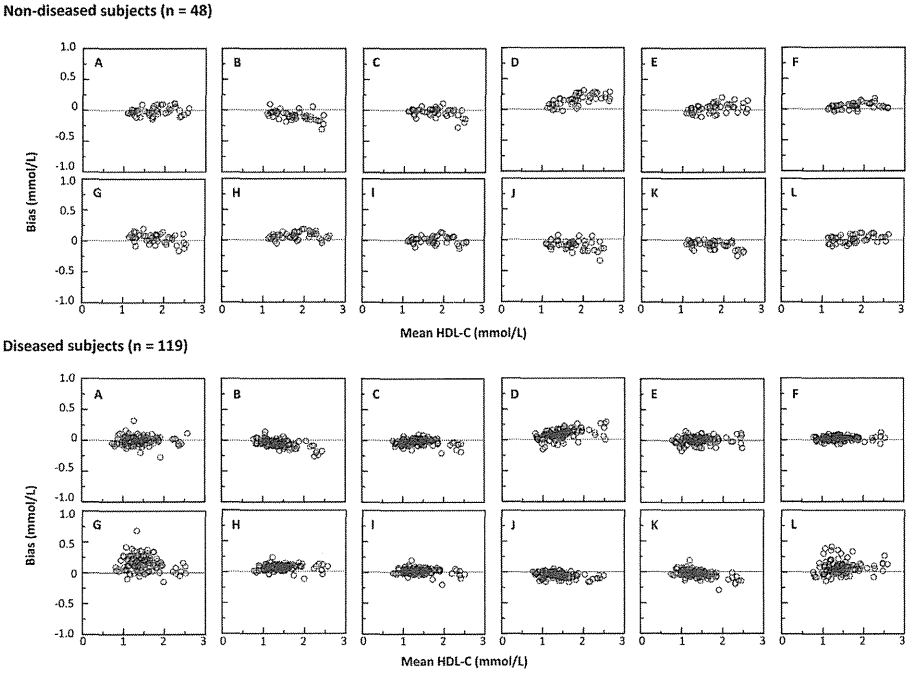

Fig. 3. Bland–Altman plots of HDL-C (H) and HDL-C (RMP) in samples from healthy and diseased subjects. The *X*-axis represents the mean HDL-C{=[HDL-C (H) + HDL-C (RMP)]/2}.

deviated slopes and Y-intercepts in samples from the healthy group, and four reagents deviated in the diseased group. Bland–Altman plots indicated the systemic proportional biases in Reagents-B, -D, -K, and -L in the diseased group (Fig. 3). Because we used HDL-C values to calculate LDL-C [25] and non-HDL-C, HDL-C values should be further standardized. We strongly suggest that the manufacturers re-evaluate the HDL-C values of their calibrators using the same fresh serum.

We conclude that HDL-C (H) concentrations measured using most of the homogeneous assay reagents (except for Reagents-D, -G, and -L) agree well with the HDL-C (RMP) concentration as determined by CDC reference method procedures in healthy and diseased subjects without extremely low HDL-C or abnormal HDL particles. The HDL-C homogeneous assays used in the described criteria enable accurate and rapid determination of HDL-C concentrations in the appropriate population.

Table 2
Accuracy of HDL-C (H) reagents evaluated with a single measurement (A) and error component analysis (B).

Subjects	Reagent											
	A	B	C	D	E	F	G	H	I	J	K	L
A: Percentage of samples that met the TE requirement of the NCEP for a single HDL-C determination[a]												
Non-diseased subjects (n = 48)	100.0	97.9	97.9	62.5	97.9	100.0	100.0	100.0	100.0	89.6	95.8	91.6
Diseased subjects (n = 119)	93.2	99.1	97.4	75.6	96.6	100.0	43.7	92.4	97.5	94.1	97.4	68.1
B: Error component analysis												
Non-diseased subjects (n = 48)												
CV_b (%)[b]	[1.2]	[0.7]	[1.2]	[1.4]	[0.9]	[0.8]	[1.2]	[1.0]	[0.9]	[1.1]	[1.0]	[2.9]
CV_e (%)	0.8	0.5	0.6	0.5	0.6	0.4	0.7	0.5	0.6	1.9	0.7	1.7
CV_d (%)	3.8	3.3	3.3	4.1	4.2	2.4	4.1	3.0	3.2	3.2	3.2	3.6
CV_t (%)	4.0	3.4	3.4	4.2	4.3	2.5	4.2	3.2	3.3	4.3	3.4	4.2
Mean bias (%) (SD)	−0.1 (3.9)	−0.9 (3.7)	−0.2 (3.5)	2.1 (4.6)	0.5 (4.2)	0.8 (2.6)	0.8 (5.0)	1.2 (3.2)	0.3 (3.2)	−1.0 (4.0)	−0.9 (3.2)	0.4 (3.7)
TE (%), for greater of positive or negative limit	8.1	−7.5	6.9	11.1	9.4	6.0	9.6	7.8	7.2	−9.3	−7.4	9.3
Diseased subjects (n = 119)												
CV_b (%)[b]	[1.2]	[0.7]	[1.2]	[1.4]	[0.9]	[0.8]	[1.2]	[1.0]	[0.9]	[1.1]	[1.0]	[2.9]
CV_e (%)	0.7	0.5	0.7	0.6	0.6	0.4	0.7	0.6	0.6	1.5	1.0	1.8
CV_d (%)	5.6	3.3	3.4	5.1	4.5	2.5	8.0	3.3	3.3	2.8	3.6	7.7
CV_t (%)	5.7	3.5	3.6	5.2	4.6	2.6	8.1	3.4	3.5	3.9	3.8	8.0
Mean bias (%) (SD)	0.3 (5.9)	−0.5 (4.2)	−0.2 (3.8)	1.8 (5.3)	0.4 (4.7)	0.8 (2.8)	3.3 (11.6)	1.6 (3.9)	0.7 (3.9)	−0.6 (3.6)	−0.2 (4.4)	1.5 (9.5)
TE (%), for greater of positive or negative limit	12.4	−7.1	7.3	13.0	10.0	6.2	21.4	8.7	7.9	−8.1	7.7	19.2

TE, total error.
Based on the method of our previous study [13], we calculated three different CV values, CV_b, CV_e and CV_d for (1) inter-assay variations, (2) intra-assay variations, and (3) variations due to subject sample-specific effects, respectively. CV_t was calculated as the square root of CV_b^2, CV_e^2 and CV_d^2.
[a] The TE requirement of the NCEP is 95% of results <13%.
[b] We determined CV_b using the pooled serum (Supplemental Table S3, Ref. [13]), and used the same CV_b values in both non-diseased and diseased groups.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2013.12.033.

Conflict of interest

Investigators of the LDL-C Study Group received no remuneration for conducting this study.

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Original Article

Predicting Coronary Heart Disease Using Risk Factor Categories for a Japanese Urban Population, and Comparison with the Framingham Risk Score: The Suita Study

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Background: The Framingham risk score (FRS) is one of the standard tools used to predict the incidence of coronary heart disease (CHD). No previous study has investigated its efficacy for a Japanese population cohort. The purpose of this study was to develop new coronary prediction algorithms for the Japanese population in the manner of the FRS, and to compare them with the original FRS.
Methods and Results: Our coronary prediction algorithms for Japanese patients were based on a large population-based cohort study (the Suita study). The study comprised 5,886 initially healthy Japanese subjects. They were followed-up for 11.8 years on average, and 213 cases of CHD were observed. A multiple Cox proportional hazard model by stepwise selection was used to construct the prediction model. The C-statistics showed that the new model had better accuracy than the original and recalibrated Framingham scores. The net reclassification improvement (NRI) by the Suita score with the inclusion of CKD was 41.2% ($P<0.001$) compared with the original FRS. The recalibration of the FRS slightly improved the efficiency of the prediction, but it was still worse than the Suita score with the CKD model. The calibration analysis suggested that the original FRS and the recalibrated FRS overestimated the risk of CHD in the Japanese population. The Suita score with CKD more accurately predicted the risk of CHD.
Conclusion: The FRS and recalibrated FRS overestimated the 10-year risk of CHD for the Japanese population. A predictive score including CKD as a coronary risk factor for the Japanese population was more accurate for predicting CHD than the original Framingham risk scores in terms of the C-statics and NRI.

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Key words:

Introduction

The Framingham Heart Study identified the classic risk factors for coronary heart disease (CHD)[1], and it developed multivariable predictive instruments, which enable clinicians to estimate the 10-year individual risk of developing CHD[2, 3]. These findings have also been widely adopted in clinical guidelines[4, 5]. However, the FRS cannot be generalized for other populations, since 99% of the Framingham cohort participants were Caucasian[5]. For example, the use of the FRS in some other populations resulted in an overestimation of the CHD risk[6-8].

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There has been relatively little attention paid to the validity of the FRS in the Japanese population, which constitutes a unique population in many aspects, with a markedly lower incidence of CHD than Western populations[9]. To our knowledge, no previous Japanese cohort study has been performed to evaluate the original and recalibrated FRS.

Several Japanese cohort studies developed risk prediction tools for Japanese patients. The NIPPON DATA 80 prediction tool has been used as the standard prediction tool in Japan[10], and has been adopted by some clinical guidelines for the stratification of risk in Japanese subjects[11]. However, the NIPPON DATA 80's outcome measure was coronary death, not the incidence of CHD. The Hisayama study predicted a composite outcome of stroke and CHD[12]. Noda's prediction score also applied to cardiac mortality[13]. The JALS study group developed a prediction tool for acute myocardial infarction (AMI), but their prediction period was relatively short (five years)[14]. The JMS-cohort study chart was also targeted for AMI, but the population was limited to rural residents[15]. These tools are all associated with some advantages and disadvantages. However, additional tools for the prediction of CHD are needed that can accurately assess the risk of the longer-term incidence of CHD in the Japanese population.

In this context, we have developed a new algorithm, named the Suita Score, for predicting the 10-year probability of developing CHD, which is based on the findings of a large population-based cohort study performed in an urban area in Japan.

Furthermore, chronic kidney disease (CKD) has recently been advocated as an independent risk factor for CHD, and patients with CKD tend to possess multiple CVD risk factors, and thus represent a major public health problem[16, 17]. A recent CHD risk assessment tool based on 2.3 million patients, the QRISK2, included CKD as a necessary component for the risk prediction[18]. Moreover, CKD patients tend to have an underestimated CHD risk based on the FRS[19]. In addition, we previously reported that CKD leads to an increased risk of both MI and stroke[20]. Hence, the objective of this study was;

1) To incorporate established classic coronary risk factors into newly developed coronary prediction algorithms for the Japanese population,

2) To compare the discriminatory properties of this approach with those of the original and recalibrated FRS

Methods

Populations

The Suita study, a cohort study of urban residents in Japan, was started in 1989. It was based on a random sampling of 12,200 Japanese residents living in Suita. As a baseline, participants between the ages of 30 and 79 years of age were randomly selected from the municipality population registry in 1989. Of these, 6485 males and females underwent regular health checkups between September 1989 and March 1994. The subjects have continued to visit the National Cerebral and Cardiovascular Center (NCVC) every two years for regular health checkups[21-24]. A total of 1,546 subjects were excluded from the study based on a past history of CHD or stroke, non-fasting blood collections, missing data or because they were lost to follow-up. The data from the remaining 5,866 participants (2,788 males and 3,078 females) were used for the analyses. This cohort study was approved by the Institutional Review Board of the National Cerebral and Cardiovascular Center.

Baseline Examinations

Blood samples were collected after the participants had fasted for at least 10 hours. The samples were centrifuged immediately, and a routine blood examination was performed that included the serum total cholesterol (TC), high density lipoprotein cholesterol (HDLC), triglycerides (TG), serum creatinine (Cre) and fasting blood glucose (FBG) levels. The blood pressure was measured three times on the right arm after five minutes of rest by well-trained physicians using a standard mercury sphygmomanometer. The average of the second and third measurements was used for the analyses. Public health nurses obtained information on the smoking and drinking habits and medical histories. To ensure comparability with the FRS, the categorization of the BP, diabetes, TC and HDL-C in this study were done in accordance with the criteria used in the FRS model3. DM was defined as an FBG level ≥ 7.0 mmol/L (126 mg/dl) and/or current use of anti-diabetic medication. Cigarette smoking was dichotomized as current versus non-current. The LDL-C was determined by the Freidewald equation.

Definition of CKD

The serum Cre level was measured using the noncompensated kinetic Jaffe′ method. The estimated glomerular filtration rate (eGFR) of each participant was calculated from Cre value and the age, using the MDRD equation below, modified with the Japanese

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coefficient (0.881)[25]:

eGFR (ml/min/1.73 m2)=0.881*186*age$^{-0.203}$*Cre$^{-1.154}$ (for males)

eGFR=0.881*186*age$^{0.203}$*Cre$^{-1.154}$*0.742 (for females).

The CKD stage was defined by the K/DOQI clinical practice guidelines[26]. CKD was categorized into Stage 3 CKD (eGFR 30-60 ml/min/1.73m^2) and Stage 4 or 5 CKD (eGFR<30 ml/min/1.73m^2).

Endpoint Determination

The follow-up method used in the Suita study has been reported previously[20-24]. The endpoints for the current follow-up study were: (1) the date of the first diagnosis of CHD (2) the date of death, (3) the date when the subject left Suita or (4) censoring by December 31, 2007.

The first step in the survey for CHD involved checking the health status of all the participants at clinical visits carried out every two years, and by yearly questionnaires sent by mail or conducted by telephone. The second step involved the review of in-hospital medical records of participants who were suspected to have developed CHD. The criteria for definite or probable acute myocardial infarction were the same as the criteria used for the MONICA project[27].

In order to complete the surveillance for fatal MI, we also conducted a systematic search of death certificates. In addition to acute myocardial infarction, the criteria for a diagnosis of CHD included sudden cardiac death within 24 hours after the onset of acute symptoms, or CHD followed by coronary artery bypass or angioplasty.

Statistical Analysis

First, we evaluated the validity of categorical variables in the Suita Score to compare them with the original FRS[3]. Then, we conducted a multiple Cox proportional hazard model using the same categories as those in the FRS. Subsequently, we developed a new CHD risk score for Japanese subjects based on the Cox model for the Suita cohort. Other risk factors were calculated using the same categories as the FRS. A stepwise selection with a *p*-value of 0.1 for backward elimination was used to select the best predictive model.

After selection of the best Cox model, we fitted the hazard functions developed by the Framingham investigators from the previously published data[6] for predicting the 10-year probability of developing CHD in the Suita cohort. The probability function was: $P=1-S(t)^{\exp(X, M)}$; $f(X, M)=\beta_1*(X_1-M_1)+\ldots+\beta_n*(X_n-M_n)$,

where S(t) is the survival rate for the mean values of the risk factors at 10 years in the Suita study; $\beta_1 \ldots \beta_n$ are the regression coefficients of the Cox model (β) shown in **Table 3**; $X_1 \ldots X_n$ represent the individual risk factor values of each study participant and $M_1 \ldots M_n$ are the mean values of the risk factors in the Suita cohort. In the recalibrated Framingham functions, the coefficients were taken from the Framingham Cox model, but the mean values from the Suita cohort were used for the risk factors and the mean incidence rates[6].

Discrimination and calibration were used to evaluate the predictive capabilities of the models. We evaluated the discriminatory ability of this model by comparing the means of the C-statistics and Bayesian information criteria (BIC). Furthermore, we measured the model improvement as indicated by the clinical reclassification of the FRS by the Suita Score, which is considered to be more important indicator for predictive ability using the net reclassification improvement (NRI)[28]. Since the inclusion of a new biomarker in a prediction tool , such as the FRS, minimally improves the predictive ability, the evaluation based on the NRI is considered to be a valid approach for evaluating the new biomarker[29]. The NRI measures the reclassification of people from one risk category to another resulting from the addition of the new risk factor to a prediction model with established risk. If all of the people end up in a more correct risk class based on the model with the new marker, the NRI is positive. We calculated the category-free NRI[30].

The third approach was calibration, which measured how closely the predicted risk fit the actual risk. The Suita participants were divided into quintiles of 10-year CHD risk predicted by the Suita score functions, the original Framingham functions and the recalibrated Framingham functions[6]. The predicted and actual risk in each quintile were compared, and the differences were assessed by the Hosmer-Lemeshow chi-square tests. The SAS software program, version 9.3. (SAS Institute Inc), and the STATA software program, version 12 (STATA Corp LP), were used for all of the statistical analyses.

Results

Population Characteristics

The number of person-years studied consisted of 75,776 (34,480 for males and 41,296 for females), with a mean follow-up period of 11.8 years. During the follow-up period, there were 213 incidents of CHD. The population characteristics are summarized in **Table 1**. The univariate Cox regression analysis

Table 1. Population characteristics of the study cohort

	Males (n=2796)	Females (n=2725)
Age (years, mean ± SD)	56.1 ± 13.3	54.5 ± 12.9
DM (%)	6	5.8
Current smoker (%)	49.67	11.91
Blood pressure (mmHg, %)		
Optimal (SBP < 120, DBP < 80)	30.74	41.68
Normal (SBP < 130, DBP < 85)	19.31	17.30
High normal (SBP < 140, DBP < 90)	17.98	15.69
Stage I HT (SBP < 160, DBP < 100)	20.39	15.94
Stage II to IV HT (SBP < 160, DBP ≥ 100)	11.59	9.40
Total cholesterol (mg/dl, %)		
< 160	10.12	6.88
160-199	39.75	30.52
200-239	37.41	39.60
240-279	10.98	18.51
≥ 280	1.74	4.49
LDL cholesterol (mg/dl, %)		
> 130	55.54	45.78
130-159	28.19	30.86
> 160	16.26	23.34
HDL cholesterol (mg/dl, %)		
< 35	11.40	3.28
35-44	28.71	16.36
45-49	15.87	12.25
50-59	23.82	29.95
≥ 60	20.20	38.14
Creatinine (mg/dl, mean ± SD)	0.91 ± 0.21	0.69 ± 0.22
eGFR (mean ± SD)	64.7 ± 24.9	90.6 ± 29.3
CKD (≥ Stage 3) (%)	46.2	11.3

LDL, Low-density lipoprotein; HDL, high-density lipoprotein;
eGFR, estimated glomerular filtration rate (ml/min/1.73 m^2); CKD, chronic kidney disease; HT, hypertension

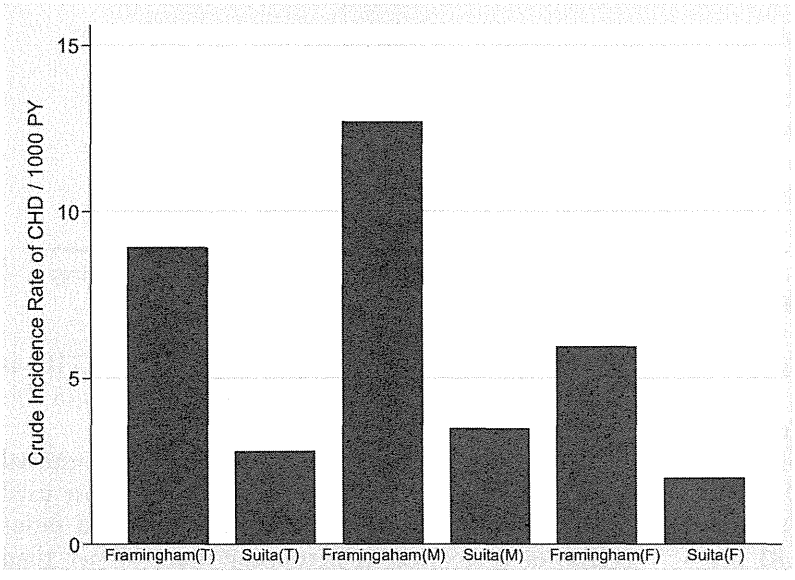

Fig. 1. The absolute risk difference of the Framingham cohort and Suita Study cohort

The Framingham cohort data were adopted from Wilson's study
CHD, coronary heart disease; PY, person-years; Framingham(T), total Framingham cohort; Framingham(M), Male Framingham cohort; Framingham(F), Female Framingham cohort; Suita(T), total Suita cohort; Suita (M), Male Suita cohort; Female Suita (F), Suita cohort

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Table 2. The multivariable-adjusted hazard ratios for coronary heart disease based on the FRS categories

MALES				
Variable	Relative Risk	*P*-value	95% CI	Framingham Cohort
Age, y	1.07	<0.001	1.05-1.09	1.05
TC, mg/dl				
<200	Reference			
200-239	1.30	0.172	0.89-1.88	1.31
≥ 240	2.15	0.001	1.38-3.34	1.9
HDL-C mg/dl				
<35	2.06	0.001	1.37-3.10	1.47
35-59	Reference			
≥ 60	0.67	0.103	0.42-1.08	0.56
Blood Pressure				
Normal (including optimal)	Reference			
High normal	1.52	0.104	0.92-2.51	1.31
Stage I hypertension	2.24	<0.001	1.45-3.46	1.67
Stage II hypertension	2.34	0.001	1.41-3.86	1.84
Diabetes (y/n)	1.39	0.234	0.81-2.40	1.5
Smoking	1.25	0.193	0.89-1.76	1.68
CKD	1.34	0.109	0.94-1.92	N.A
FEMALES				
Variable	Relative Risk	*P*-value	95% CI	Framingham Cohort
Age, y	1.10	<0.001	1.07-1.13	1.04
TC, mg/dl				
<200	Reference			
200-239	0.58	0.097	0.30-1.10	1.51
≥ 240	1.38	0.272	0.78-2.46	1.72
HDL-C mg/dl				
<35	1.94	0.102	0.88-4.31	2.02
35-59	Reference			
≥ 60	1.04	0.881	0.61-1.79	0.58
Blood Pressure				
Normal (including optimal)	Reference			
High normal	1.60	0.222	0.75-3.38	1.3
Stage I hypertension	1.82	0.089	0.91-3.61	1.73
Stage II hypertension	3.86	<0.001	1.99-7.48	2.12
Diabetes (y/n)	2.59	0.013	1.23-5.49	1.77
Smoking	3.22	<0.001	1.74-5.97	1.47
CKD	1.38	0.247	0.80-2.40	N.A

FRS, Framingham risk score; All variables were adjusted for all FRS variables by a Cox proportional hazard model. CKD, chronic kidney disease; 95% CI, 95% confidence interval; N.A, not available

indicated all variables in FRS were statistically significant (data not shown).

Fig. 1 depicts the difference in the absolute risk for CHD between the Framingham cohort and Suita study cohort. The crude incidence rate of CHD in the original Framingham cohort was 8.94 per 1000 person-years, while that of the Suita cohort was 2.81 per 1000 person-years. The risk of developing CHD is nearly one-third of both the males and females in this study cohort.

The results of the multiple Cox proportional hazard model using the same categories as those used in the FRS are shown in **Table 2**. All hazard ratios (HRs) of categorical hypertension were higher than