Blood biomarkers

BRHS 20 year follow-up (Q20)

1998 - 2000

Fasting blood samples

At the 20 year follow-up physical examination in 1998-2000 (Q20), the BRHS study participants were asked to fast for a minimum of 6 hours, during which they were instructed to drink only water and to attend for measurement at a prespecified time between 0800 and 1800 h. All men were asked to provide a blood sample, collected using the Sarstedt Monovette system. The samples were frozen and stored at −20°C on the day of collection and transferred in batches for storage at −70 °C until analysis, performed after no >1 freeze-thaw cycle.

The blood sampling procedure is described in section 4.2.6 and the handling of the blood samples is described in sections 5.1 to 5.5 of the 20 year follow-up physical examination protocol. (***BRHS 1998-2000 (Q20) 20yr follow-up Physical examination protocol.pdf***)

Blood marker adjustments

** Adjusted blood markers: A number of blood markers (Glucose, insulin, triglycerides and LDL) were adjusted for fasting duration (using the time the participants recorded as having something to eat or drink other than water – (*question 20.0 in BRHS 1998-2000 20 year follow-up survey Main Qr Q20*). These blood markers are available both in unadjusted and adjusted form. The method of adjustment is described in *Emberson J,Whincup PH, Walker M , Thomas M,Alberti KGMM. Biochemical measures in a population-based study: effect of fasting duration and time of day. Ann Clin Biochem 2002; 39: 493- 501* and in the BRHS document: *BRHS Q20 Blood marker Adjustment Report and SAS code by JEmberson.pdf* (Appendix 1).

A list of all BHRS bloods markers for 20 year follow-up in 1998-2000(Q20) can be found in the table below followed by the laboratory methods used. ** indicates the blood marker has been adjusted for fasting duration.

Appendix 1 : BRHS Q20 Blood marker Adjustment Report and SAS code by JEmberson.pdf + reference

Blood biomarkers - BRHS 1998-2000 (Q20) 20 year follow-up

Blood marker	units	Method section	BRHS variable name	Mean	SD	Min	Max	N	N Miss	Data access
Activated partial thromboplastin time (aPTT)	s	S.3	q20appt	30.98	3.63	22.4	79.4	4079	173	yes
Activated protein C (APC resistance)	ratio	S.3	q20apc	3.29	0.56	1.73	6.69	4050	202	yes
Adiponectin	µg/mL	S.5	q20adiponectin	8999.7	7319.7	6.15	66984.93	4046	206	yes
Alanine Transaminase (ALT)	u/l	S.1.3	q20alt	17.49	10.09	1	201	4034	218	yes
Albumin	g/l	S.1.6	q20alb	44.15	2.75	29	54	4034	218	yes
Alkaline Phosphatase (ALP)	u/l	S.1.5	q20alk_phos	85.82	41.56	16	1194	4034	218	yes
Aspartame Transaminase (AST)	u/l	S.1.3	q20ast	24.25	10.31	6	268	4034	218	yes
Bilirubin	µmol/L	S.1	q20bil	10.16	5.67	1	209	4021	231	yes
Calcium	mmol/L	S.1.6	q20ca	2.43	0.09	1.83	3.7	4033	219	yes
Carboxyhemoglobin	%	S.13	q20cohb	0.83	1.15	0	10.6	4025	227	yes
Cholesterol	mmol/l	S.1.1	q20chol	6	1.08	2.6	11.6	4031	221	yes
Copeptin	pmol/L	S.6	q20copeptin	5.43	5.29	0.89	91.91	3713	539	yes
Corrected Calcium	mmol/L	S.1.6	q20corr_ca	2.35	0.08	2.02	3.66	4033	219	yes
Cotinine	ng/ml	S.12	q20cotinine	41.96	107.49	0.05	844.2	3900	352	yes
C-Reactive protein (CRP)	mg/L	S.3	q20crp	3.53	6.86	0.18	120	4056	196	yes
Creatinine	µ mol/l	S.1	q20cre	98.15	21.19	39	531	4034	218	yes
Cysteine	mg/L	S.8	q20cys	312	37.91	165.2	484.8	3921	331	yes
Cysteinylglycine (CysGly)	µmol/L	S.8	q20cg	32.9	6.02	7.6	62.8	3921	331	yes
D-dimer	ng/ml	S.3	q20ddim	133.58	210.74	3	2852	4079	173	yes
eGFR $q20egfr=32788 \times q20cre^{(-1.154)} \times q20age^{(-0.203)}$		S.13	q20egfr	72.37	12.79	9.86	203.56	4034	218	yes
Factor IX	IU/dL	S.3	q20f9	131.3	25.85	19	223	4078	174	yes
Factor VII	IU/dL	S.3	q20f7	117.23	26.43	9	215	4073	179	yes
Factor VIII	IU/dL	S.3	q20f8	132.38	31.81	15	332	4077	175	yes
Fibrinogen	g/L	S.3	q20fib	3.27	0.74	0.49	8.16	4080	172	yes
Nephelometric fibrinogen	g/L	S.3	q20nephfib	3.17	0.72	0	8.59	4026	226	yes
Gamma-glutamyl transferase (gamma-GT, GGT)	u/l	S.1.3	q20ggt	36.83	55.05	7	1879	4034	218	yes
**Glucose(adjusted)	mmol/l	S.1.2a	q20dglu	6.03	1.89	2.55	27.6	4032	220	yes
*Glucose(unadjusted)	mmol/l	S.1.2	q20glu	6.06	1.91	2.5	27.6	4032	220	yes
Glycated Haemoglobin (HbA1c)	%	S.15.1	q20hba1c	5.03	0.93	1.2	12.9	4039	213	yes
Haematocrit	l/l	S.15	q20hct	0.45	0.03	0.28	0.66	4036	216	yes
Haemoglobin(hb)	g/l	S.15	q20hb	14.58	1.18	7.1	20.2	4036	216	yes
HDL cholesterol	mmol/l	S.1.1	q20hdl	1.32	0.34	0.3	3.3	4006	246	yes

** blood marker has been adjusted for fasting duration

Blood marker/cont.	units	Method section	BRHS variable name	Mean	SD	Min	Max	N	N Miss	Data access
Homocysteine	µmol/L	S.8	q20hcy	13.48	6.47	5.3	128.7	4021	231	yes
**Insulin(adjusted)	pmol/L	S.2a	q20dinsul	11.19	14.89	1.12	522	4023	229	yes
*Insulin(unadjusted)	pmol/L	S.2	q20rinsul	10.93	15.11	1	522	4023	229	yes
Interleukin-6 (IL-6)	pg/ml	S.3	q20il6	3.18	2.95	0.06	16	4050	202	yes
Leptin	ng/ml	S.7	q20leptin	12.34	13.05	0	173.7	4018	234	yes
**LDL cholesterol(adjusted)	mmol/l	S.1.1a	q20dldl	3.89	0.97	0.71	9.37	3974	278	yes
*LDL cholesterol(unadjusted)	mmol/l	S.1.1	q20ldl	3.87	0.97	0.71	9.1	3974	278	yes
Magnesium (Mg)	mmol/L	S.1.6	q20mg	0.81	0.07	0.48	1.06	4031	221	yes
Mean Cell Haemoglobin Concentration (MCHC)	g/dl	S.15	q20mchc	32.37	1.19	25.84	37.11	4036	216	yes
Mean Cell Haemoglobin (MCH)	pg	S.15	q20mch	30.08	1.84	17	40	4037	215	yes
Mean Cell volume (MCV)	fl	S.15	q20mcv	92.97	5.27	65.7	123.1	4036	216	yes
Mean Platelet volume (MPV)	fl	S.15	q20mpv	8.4	1.73	3.6	14.9	3889	363	yes
MR-proADM	nmol/L	S.4	q20MRproADM	0.6	0.21	0.05	6.24	4050	202	yes
NT-proBNP	pg/ml	S.4	q20bnp	238.29	535.72	5	9771	3784	468	yes
Parathyroid Hormone (Pth)	pg/mL	S.11	q20PTH	48.56	21.27	14.5	620.6	3889	363	yes
Phosphate	mmol/l	S.1.5	q20phos	1.16	0.16	0.47	2.1	4012	240	yes
Plasma Viscocity	mPa s	S.3	q20pv	1.29	0.08	1.1	2.33	4013	239	yes
Platelets	109 /l	S.15	q20plts	235.58	63.44	27	899	4036	216	yes
Potassium (K)	mmol/l	S.1	q20k	4.43	0.37	3.1	6.3	4013	239	yes
Protein	g/l	S.1	q20prot	76.96	4.7	56	143	4034	218	yes
Red blood cell count (RBC)	1012 /l	S.15	q20rbc	4.86	0.4	2.56	6.7	4036	216	yes
Sodium (Na)	mmol/l	S.1.4	q20na	139.62	2.74	125	150	4034	218	yes
Tissue plasminogen activator(tPA)	ng/ml	S.3	q20tpa	11.08	4.44	1.5	36.8	4083	169	yes
**Triglycerides(adjusted)	mmol/l	S.1.1a	q20dtrig	1.86	1.08	0.37	16.66	4032	220	yes
*Triglycerides(unadjusted)	mmol/l	S.1.1	q20trigs	1.84	1.09	0.35	15.95	4032	220	yes
Troponin T(TnT)	pg/ml	S.4	q20TNT	14.11	22.25	2.99	1301	4046	206	yes
Urate	mmol/l	S.1.7	q20urate	0.38	0.08	0.14	0.8	4034	218	yes
Urea	mmol/l	S.1	q20urea	6.05	1.7	1.8	24.3	4034	218	yes
Vitamin C	µmol/L	S.9	q20vit_c	30.22	27.35	0	447.92	3948	304	yes
Vitamin D,	nmol/L	S.10	q20Total_VitD	20.01	9.24	2	120	3799	453	yes
Vitamin E	mmol/L	S.9	q20vit_e	33.59	11.95	0.64	118.65	3779	473	yes
Von Willebrand factor (VWF)	IU/ml	S.3	q20vwf	139.96	46.19	23	382	4083	169	yes
White blood cell count (WBC)	109/L	S.15	q20wbc	7.11	2.18	2.7	38.6	4037	215	yes

** blood marker has been adjusted for fasting duration

S.0 Blood biomarkers - Laboratory methods and reference papers

S.1 Biochemistry variables

Measured at the Department of Chemical Pathology, Royal Free Hospital, London (Prof Mike Thomas).
Biochemistry variables including S.1.1 – S.1.7 were measured using a Hitachi 747 automated analyser.

S.1.1 Lipids

All blood samples were separated and frozen on the day of collection and transferred to central laboratories for analysis.

Total cholesterol, HDL cholesterol and Triglyceride were measured using a Hitachi 747 automated analyser. Total and HDL cholesterol were analysed using the methods of Siedel [1] and Sugichi [2] respectively. **LDL-cholesterol** values were calculated using the Friedrickson-Friedwald equation.

1. Siedel J, Hagele EO, Ziegenhorn J, Wahlefield AW. Reagent for the enzymatic determination of serum total with improved lipolytic efficiency. Clin Chemistry 1983; 29: 1075-1080.
2. Sugishi H, Uji Y, Okabe H, Uekema K, Kjayahar N. Direct measurement of high-density lipoprotein cholesterol in serum with polyethylene glycol modified enzymes and sulphated alpha-cyclodextrin. Clin Chemistry 1995; 41: 717-723.
3. Emberson J,Whincup PH, Walker M , Thomas M,Alberti KGMM. Biochemical measures in a population-based study: effect of fasting duration and time of day. Ann Clin Biochem 2002; 39: 493- 501

S.1.1a ** Triglyceride and **LDL (adjusted for for differences between participant's fasting duration)

1. Emberson J,Whincup PH, Walker M , Thomas M,Alberti KGMM. Biochemical measures in a population-based study: effect of fasting duration and time of day. Ann Clin Biochem 2002; 39: 493- 501
2. BRHS document: BRHS Q20 Blood marker Adjustment Report and SAS code by JEmberson.pdf

S.1.2 Glucose

Plasma glucose was measured using the method of Trinder [1] using a Falcor 600 automated analyser.

1. Trinder P. Determination of blood glucose using 4-aminophenazone as oxygen acceptor. J Clin Pathology 1969; 22: 246.
2. Emberson J,Whincup PH, Walker M , Thomas M,Alberti KGMM. Biochemical measures in a population-based study: effect of fasting duration and time of day. Ann Clin Biochem 2002; 39: 493- 501

S.1.2a **Glucose (adjusted for for differences between participant's fasting duration)

1 Emberson J,Whincup PH, Walker M , Thomas M,Alberti KGMM. Biochemical measures in a population-based study: effect of fasting duration and time of day. Ann Clin Biochem 2002; 39: 493- 501

2 BRHS document: BRHS Q20 Blood marker Adjustment Report and SAS code by JEmberson.pdf

S.1.3 Hepatic enzymes

Hepatic enzymes including GGT, ALT and AST, were measured using a Hitachi 747 automated analyzer.

Gamma-GT

A commercial assay supplied by Roche Diagnostics for use on Hitachi analysers. The method is standardised against the original kinetic method of Szasz but uses a water-soluble substrate L-gamma-glutamyl-3-carboxy-4-nitroanilide. Gamma-glutamyltransferase transfers the gamma-glutamyl group of this substrate to glycylglycine. the amount of 5-amino-2-nitrobenzoate liberated is proportional to the GGT activity and can be determined spectrophotometrically.

1. Szasz, G, Persjin, JP et.al. Z Klin Chem Klin Biochem (1974) 12 : 228.
2. Persijn JP, van der Slik W. A new method for the determination of gamma-glutamyltransferase in serum. J Clin Chem Clin Biochem. 1976 Sep;14(9):421-7.

ALT, AST

A commercial assay supplied by Roche Diagnostics for use on Hitachi analysers. The method is derived from the recommended IFCC reference method. The enzyme ALT catalyses the equilibrium reaction between alpha-ketoglutarate and L-alanine with that of L-glutamate and pyruvate. The increase in pyruvate is determined in an indicator reaction catalysed by lactate dehydrogenase in which NADH is oxidised to NAD+. The rate of the photometrically determined NADH decrease is directly proportional to the rate of formation of pyruvate and hence the ALT activity.

1. Bergmeyer HU, Horder M, Rej R. International Federation of Clinical Chemistry (IFCC) Scientific Committee, Analytical Section: approved recommendation (1985) on IFCC methods for the measurement of catalytic concentration of enzymes. Part 3. IFCC method for alanine aminotransferase (L-alanine: 2-oxoglutarate aminotransferase, EC 2.6.1.2). J Clin Chem Clin Biochem. 1986 Jul;24(7):481-95.

S.1.4 Sodium

Sodium was measured by an ion selective electrode.

1. Wannamethee SG, Shaper AG, Lennon L, Papacosta O, Whincup P. Mild hyponatremia, hypernatremia and incident cardiovascular disease and mortality in older men: A population-based cohort study.Nutr Metab Cardiovasc Dis. 2016 Jan;26(1):12-9

S.1.5 Phosphate, Alkaline Phosphatase (ALP)

Serum phosphate and ALP were both analysed on a Hitachi autoanalyser using colorimetric assays (Roche). The serum phosphate assay was based on the detection of ammonium phosphomolybdate; the ALP assay was based on the detection of p-nitrophenol released by ALP activity.

1. Wannamethee SG, Sattar N, Papacosta O, Lennon L, Whincup PH. Alkaline phosphatase, serum phosphate, and incident cardiovascular disease and total mortality in older men. Arterioscler Thromb Vasc Biol. 2013 May;33(5):1070-6.

S.1.6 Magnesium, Albumin, Calcium

Measured with an enzymatic colorimetric assay using a Hitachi 747 automated analyser.

S.1.7 Urate

Urate was measured with an enzymatic colorimetric assay using a Hitachi 747 automated analyser.

S.2 Insulin

Measured at the Department of Diabetes, University of Newcastle-on-Tyne (Prof G Alberti)
Serum insulin was measured using an enzyme-linked immunosorbent assay ELISA which does not cross-react with proinsulin. [1]

1. Andersen L, Dinesen B, Jorgensen PN, Poulsen F, Roder ME. Enzyme immunoassay for intact human insulin in serum or plasma. Clin Chemistry 1993; 39: 578-582.
2. Emberson J,Whincup PH, Walker M , Thomas M,Alberti KGMM. Biochemical measures in a population-based study: effect of fasting duration and time of day. Ann Clin Biochem 2002; 39: 493- 501

S.2a ** Insulin (adjusted for differences between participant's fasting duration)

1. Emberson J,Whincup PH, Walker M , Thomas M,Alberti KGMM. Biochemical measures in a population-based study: effect of fasting duration and time of day. Ann Clin Biochem 2002; 39: 493- 501
2. **BRHS document: BRHS Q20 Blood marker Adjustment Report and SAS code by JEmberson.pdf**

S.3 Haemostatic and inflammatory variables

Measured at the University of Glasgow (Prof G Lowe)

Haemostatic: aPTT, APC, D-dimer, Factor VII, Factor VIII, Factor IX, tPA, vWf
Inflammatory: Fibrinogen, Nephelometric fibrinogen, Plasma viscosity, CRP, IL-6

At the 20-year examination, blood was anticoagulated with K2 EDTA (1.5 mg mL−1) for measurement of plasma viscosity at 37 °C in a semi-automated capillary viscometer (Coulter Electronics, High Wycombe, UK). Blood was also anticoagulated with 0.109 m trisodium citrate (9:1 v:v) for measurement of clottable fibrinogen (Clauss method); as well as coagulation factors (F)VII, VIII and IX; activated partial thromboplastin time (APTT) and activated protein C (APC) ratio (measured by the APTT-based method) in an MDA-180 coagulometer (Organon Teknika, Cambridge, UK). Plasma levels of t-PA antigen and D-dimer were measured with enzyme-linked immunosorbent assays (ELISA) (Biopool AB, Umea, Sweden) as was VWF antigen (Dako, High Wycombe, UK). C-reactive protein (CRP) was assayed by ultra-sensitive nephelometry (Dade Behring, Milton Keynes, UK). IL-6 was assayed using a high-sensitivity ELISA (R & D Systems, Oxford, UK). [1]

1. Wannamethee SG, Whincup PH, Shaper AG, Rumley A, Lennon L, Lowe GD. Circulating inflammatory and hemostatic biomarkers are associated with risk of myocardial infarction and coronary death, but not angina pectoris, in older men. J Thromb Haemost. 2009 Oct;7(10):1605-11.
2. Rumley A, Emberson JR, Wannamethee SG, Lennon L, Whincup PH, Lowe GD. Effects of older age on fibrin D-dimer, C-reactive protein, and other hemostatic and inflammatory variables in men aged 60-79 years. J Thromb Haemost. 2006 May;4(5):982-7.

S.4 Cardiac markers

Measured at the University of Glasgow (Dr Paul Welsh)

NT-proBNP, hsTnT, and MR-proADM

NT-proBNP and hsTnT were measured in plasma samples on an automated clinically validated immunoassay analyzer (e411, Roche Diagnostics, Burgess Hill, United Kingdom) using the manufacturers' calibrators and quality control reagents. MR-proADM was measured on an automated B.R.A.H.M.S Kryptor Compact plus (Thermo Fisher Scientific Hemel Hempstead, United Kingdom).[1]

1. Welsh P, Hart C, Papacosta O, Preiss D, McConnachie A, Murray H, et al. Prediction of Cardiovascular Disease Risk by Cardiac Biomarkers in 2 United Kingdom Cohort Studies: Does Utility Depend on Risk Thresholds For Treatment? Hypertension. 2016;67(2):309-15. Epub 2015/12/17.

Other blood markers

S.5 Adiponectin

Measured at the University of Glasgow (Prof Naveed Sattar)

1. Sattar N, Wannamethee G, Sarwar N, Tchernova J, Cherry L, Wallace AM, Danesh J, Whincup PH. Adiponectin and coronary heart disease: a prospective study and meta-analysis.Circulation. 2006 Aug 15;114(7):623-9.

S.6 Copeptin

Measured at the University of Glasgow (Prof Naveed Sattar)

1. Wannamethee SG, Welsh P, Papacosta O, Lennon L, Whincup PH, Sattar N. Copeptin, Insulin Resistance, and Risk of Incident Diabetes in Older Men. Clin Endocrinol Metab. 2015 Sep;100(9):3332-9.

S.7 Leptin

Measured at the University of Glasgow (Prof Naveed Sattar)

1. Sattar N, Wannamethee G, Sarwar N, Chernova J, Lawlor DA, Kelly A, Wallace AM, Danesh J, Whincup PH. Leptin and coronary heart disease: prospective study and systematic review. J Am Coll Cardiol. 2009 Jan 13;53(2):167-75.

S.8 Homocysteine, Cysteine and Cysteinyl-glycine

Measured in Tromso, Norway (Dr Ueland)

S.9 Plasma Vitamin C and Vitamin E

Measured at the Department of Biochemistry, Royal Free Hospital, London (Prof Bruckdorfer)

Plasma vitamin C and vitamin E were measured with high performance liquid chromatography using ultraviolet and fluorescent detection in plasma. For vitamin C, plasma extracts were treated with metaphosphoric acid at the point of collection and were then snap frozen with dry ice (1,2).

1. Rice-Evans, Diplock CAA, Symons MCR. Techniques in free radical research. In: Burdon RH, Vanknippenberg PH, eds. Laboratory techniques in biochemistry and molecular biology. Amsterdam: Elsevier, 1991: 185–206.
2. Jennings PE, Chirico S, Jones AF, Lunec J, Barnett AH. Vitamin C metabolites and microangiopathy in diabetes mellitus. Diabetes Res 1987; 6: 151–4.
3. Wannamethee SG, Bruckdorfer KR, Shaper AG, Papacosta O, Lennon L, Whincup PH. Plasma vitamin C, but not vitamin E, is associated with reduced risk of heart failure in older men. Circ Heart Fail. 2013 Jul;6(4):647-54.

S.10 Total vitamin D

Measured at the University of Glasgow (Prof Naveed Sattar)

Total vitamin D (25OHD2 plus 25OHD3) was measured using a gold-standard liquid chromatography–tandem mass spectrometry method following an automated solid-phase extraction procedure [1]. Measurements were made in ng/ml and converted into nmol/l. The lower limit of sensitivity was 10 nmol/l. PTH was measured by electrochemiluminescence using a clinically validated assay for intact PTH [1].

1. Wannamethee SG, Welsh P, Papacosta O, Lennon L, Whincup Peter H, Sattar N. Elevated parathyroid hormone, but not vitamin D deficiency, is associated with increased risk of heart failure in older men with and without cardiovascular disease. Circ Heart Fail 2014; 7: 732–9.

S.11 Parathyroid hormone (PTH)

Measured at the University of Glasgow (Prof Naveed Sattar)

Plasma PTH was measured by electrochemiluminescence using a clinically validated assay for intact PTH on the Elecsys 2010 (Roche Diagnostics) using the manufacturer's calibrators and controls.[1]

1. Wannamethee SG, Welsh P, Papacosta O, Lennon L, Whincup Peter H, Sattar N. Elevated parathyroid hormone, but not vitamin D deficiency, is associated with increased risk of heart failure in older men with and without cardiovascular disease. Circ Heart Fail 2014; 7: 732–9.

S.12 Cotinine

Measured at New Cross Hospital, London (C Feyerabend)

Liquid chromatography tandem mass spectrometry was used to assay cotinine in serum samples [1]

1. Jefferis BJ, Lowe GD, Welsh P, Rumley A, Lawlor DA, Ebrahim S, Carson C, Doig M, Feyerabend C, McMeekin L, Wannamethee SG, Cook DG, Whincup PH. Second hand smoke (SHS) exposure is associated with circulating markers of inflammation and endothelial function in adult men and women. .Atherosclerosis. 2010 Feb;208(2):550-6.

S.13 Carboxyhaemoglobin

Measured at the Whittington Hospital, London.

COHb was measured using a co-oximeter (AVL Medical Instruments, Ltd) which was calibrated with each batch of samples and was registered in an external quality assurance programme.

Whincup PH, Papacosta O, Lennon L, Haines A . Carboxyhaemoglobin levels and their determinants in older British men BMC Public Health. 2006 Jul 18;6:189.

S.14 eGFR

eGFR(q20egfr) =32788 x q20cre $^{(-1.154)}$ x q20age $^{(-0.203)}$ where q20cre **is creatinine** and q20age is **age**

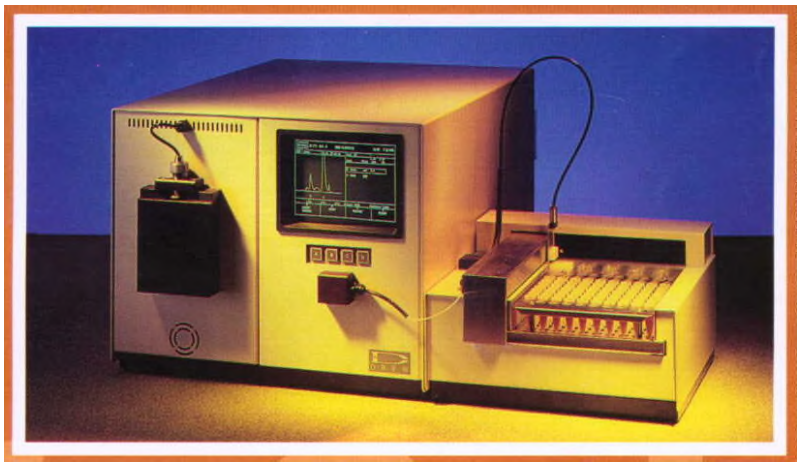
S.15 Routine Haematology

Routine haematology analyses were carried out at the Dept of Haematology, Whittington Hospital, London.

Blood was anticoagulated with K_2 EDTA (1.5 mg mL^{-1}) for measurement of haematocrit, white cell count, and platelet count in an automated cell counter.

S.15.1 Glycated Haemoglobin (HbA1c)

Drew Scientific HbGold Analyser



Intended use

The Drew Scientific HbGold Analyser, associated reagent kit and controls are intended for the in vitro measurement of HbA1c, HbA2 and the identification of haemoglobin variants in EDTA anticoagulated whole blood samples.

Principle

The instrument utilises automated cation exchange chromatography in conjunction with gradient elution to separate human haemoglobin subtypes and variants from haemolysed whole blood.

Results are automatically displayed on an integral screen as a chromatogram and they can be also be stored by an internal computer, transmitted by a standard RS232 port or printed on the standard dot-matrix printer supplied. Data can also be archived to floppy disk.

System

The Drew Scientific HbGold Analyser includes a 100 position cooled Autosampler which handles pre-diluted blood samples and automatically introduces them to the analyser.

For HbA1c measurement the analysis time is 5.5 minutes, for HbA2 and variants is 7 minutes and there is a 13-minute high-resolution assay available for further haemoglobin investigations.

The software allows the user to change between the above assays even within the same batch, using a single “Gold” column and reagent kit.

Precision

The maximum co-efficient of variance is 5% for the HbA1c and HbA2 assays.

Wed. 25 April 2000 . Wed. 27 June 2001

APPENDIX 1

Adjustment of Continuous Variables for Fasting Duration and Time of Appointment.

Jon Emberson – 8/3/2001

SUMMARY

An adjustment for differences between subject's fasting durations was carried out for total cholesterol, LDL cholesterol, triglycerides, insulin and glucose. A cut off point of 6 hours was used to define a successful faster from a non successful one. For total cholesterol, non-fasters had their total cholesterol values adjusted upwards by an amount based on their diabetic status only. For LDL cholesterol and triglycerides, diabetics and non-diabetics were adjusted separately based on the fitting of two separate models. In both cases, the non fasters were adjusted to amount synonymous with the fasters. For insulin and glucose levels, it became apparent that there was a diurnal effect of fasting time, with subjects with either a short (<6 hours) or medium duration fast (14 hours) having markedly higher readings than other subjects. This was almost certainly due to miss-reporting of true fasting duration times by subjects whose appointment was in the afternoon. Therefore, for these responses, models were built based on *both* fasting duration and time of appointment. All subjects were subsequently standardised to that of a 6 hour faster with a 1P.M appointment. Again, models were fitted separately for both diabetics and non-diabetics.

VARIABLES OF INTEREST

The variables considered to potentially vary by fasting duration times were total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, fibrinogen, insulin and glucose. At the onset, diabetics were considered separately from non-diabetics, as they were the only group of subjects that were not asked to fast for a period of at least six hours before their examination.

TRANSFORMATION OF VARIABLES

All the measures apart from total cholesterol were skewed to some degree. The square root transformation was applied to HDL and LDL cholesterol, and to fibrinogen, and the log transform applied to triglycerides and insulin. A double log transformation was applied to glucose levels, due to the extent of skewness observed in this data.

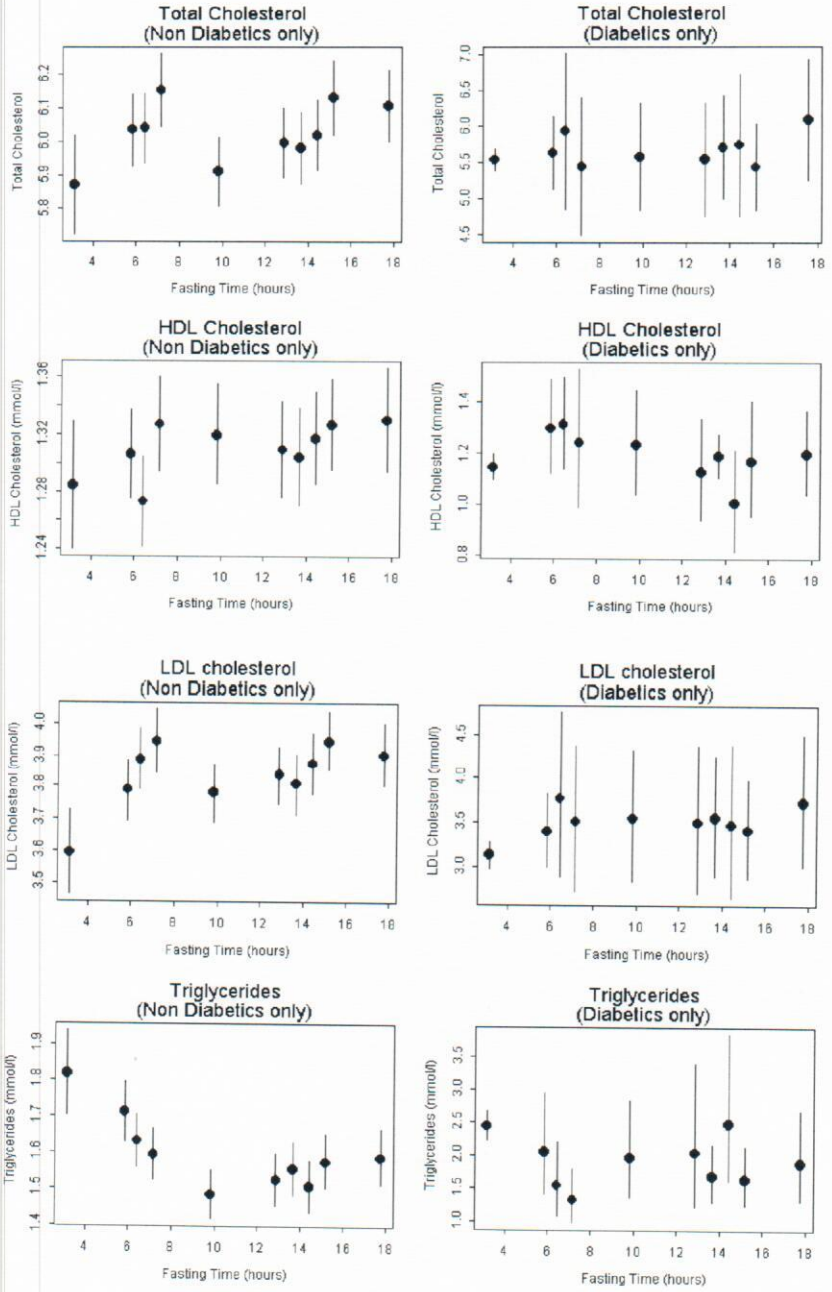
CALCULATION OF FASTING TIMES

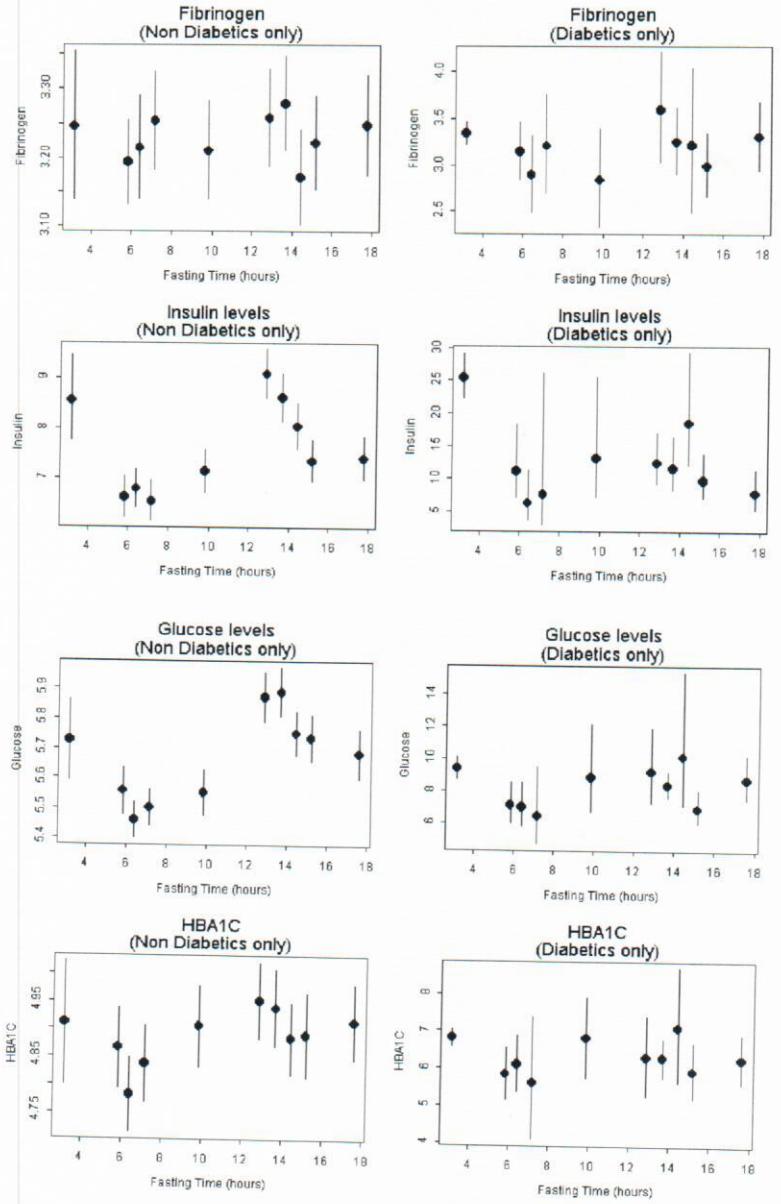
Fasting duration times were calculated for each subject attending the 20 year follow up as the difference in hours from the time recorded on the datasheet (when the sample was taken) and the time that the subject claimed to have last eaten (taken from the blue questionnaire). In cases where the subject last ate yesterday, but gave no actual time estimate, the time was estimated as 9pm, and the fasting time was calculated accordingly.

EXPLORATORY ANALYSIS – RELATIONSHIPS WITH FASTING DURATION

Fasting times were available for 4220 of the 4252 subjects that attended the twenty year follow up. Fasting times ranged from 5 minutes to 33 hours. The mean (standard deviation) fasting time for diabetics was 6.3 hours (5.5 hours), and was 11.1 hours (4.7 hours) for non-diabetics. 87% of non diabetics successfully fasted for the requested period of 6 hours. 33% of diabetics claimed to have fasted for at least 6 hours.

Subjects were categorized into groups defined by the deciles of fasting duration. For each group and each measure, 95% confidence intervals for the mean were calculated and plotted in an attempt to view any possible threshold relationships with levels of fasting duration. These are shown below and overleaf for both diabetics (right) and non-diabetics (left).





ANALYSIS BY FASTING DURATION AND DIABETIC STATUS: 2-WAY ANOVA

Preliminary analyses were carried out to assess whether or not it appeared that fasting status (i.e. whether a subject successfully fasted for a period of 6 hours or not) affected the blood measurements taken. Two way analysis of variance was used to examine the effects of fasting status and diabetic status on each of the measures.

Whether or not a person was a diabetic affected all the measures except fibrinogen. Fasting status was found to significantly affect total cholesterol, LDL cholesterol, triglycerides, insulin and glucose levels, but not HDL cholesterol or fibrinogen. Furthermore, for LDL cholesterol, triglycerides, insulin and glucose, the effect of being a faster rather than a non faster differed significantly in magnitude by diabetics status.

It was therefore decided (Peter & Jon) that an adjustment for fasting durations would be necessary for total cholesterol, LDL cholesterol, triglycerides, insulin and glucose.

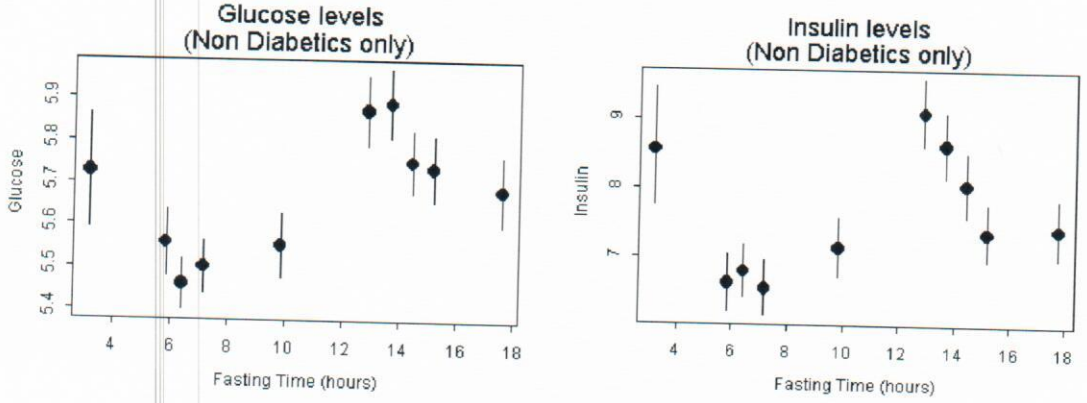
ADJUSTMENT OF TOTAL CHOLESTEROL, LDL CHOLESTEROL AND TRIGLYCERIDES

In fitting models to adjust for fasting status for these variables, it was desirable to keep the models simple.

For total cholesterol, LDL cholesterol and triglycerides, linear regression was used to quantify the differences by diabetic status and fasting status. Since the interaction between diabetic status and fasting status was found to be significant for LDL cholesterol and triglycerides, separate models were fitted for the diabetics and the non diabetics, leaving fasting status as the only explanatory variable. The coefficients from these four analyses were then used to respectively adjust the measures of the non fasters to an amount synonymous with the fasters. For total cholesterol, one model was fitted containing fasting status and diabetic status as the explanatory variables. Non fasters were adjusted to the level of fasters, whilst preserving the differences between diabetics and non diabetics.

INSULIN AND GLUCOSE – A PERIODIC EFFECT?

By examination of insulin and glucose levels for non diabetics, there appeared to be a periodic effect of fasting time where peaks were observed at both the lowest level of fasting, but also for fasting levels around 13 hours. This is clearly indicated below.



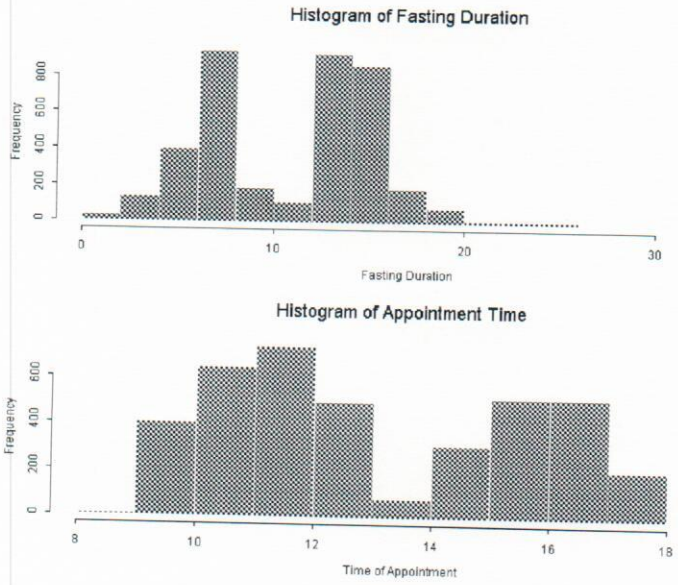
Further examination of the subjects contributing to these data revealed that reported fasting duration was strongly related to time of appointment, with subjects having morning appointments being more likely to report long fasting times (over night) than subjects with afternoon appointments. The second peaks observed for both insulin and glucose levels at 13 hours fasting duration may then be attributable to subjects failing to report accurate fasting times, perhaps they had a drink for breakfast which they failed to report. These peaks may therefore be due to missreporting of fasting times rather than any true periodic effect.

Nevertheless, this needs to be accounted for in an adjustment of these variables[1].

[1] Providing that the adjustment is carried out based on a suitable model derived from the data, it is irrelevant how accurate the fasting times actually are.

TRANSFORMATION OF FASTING DURATION & APPOINTMENT TIME

Both fasting time and time of appointment are bi-modal, as can be seen below. For appointment time, the modes correspond to the peak hours in the morning and afternoon, whilst for fasting duration, the explanation is probably attributable to missclassification. In order to model linearly with these variables certain transformations were carried out.



Fasting time was categorized into one of two intervals, [0,10), or [10, 20)[2], creating a dichotomous variable. The fasting time was also divided modulo 10, creating a continuous variable defined on the half open interval [0, 10). The aim of this was to replace the original data with two variables, a continuous variable and a binary variable[3].

For time of appointment, a similar calculation was performed. All appointments were categorized as either morning or afternoon appointments, where afternoon appointments began at 1 P.M. This binary variable then defines the time of appointment as belonging to one of the intervals [8, 13) or [13, 18), using the 24 hour clock notation. By shifting these intervals to the origin and performing a division modulo 5, we have a continuous variable defined on the half open interval [0, 5)[4].

The purpose of these transformations was to keep all the information contained in the fasting time and time of appointment, whilst facilitating linear modeling of the variables. The transformed continuous variables are shown overleaf, on the square root scale[5].

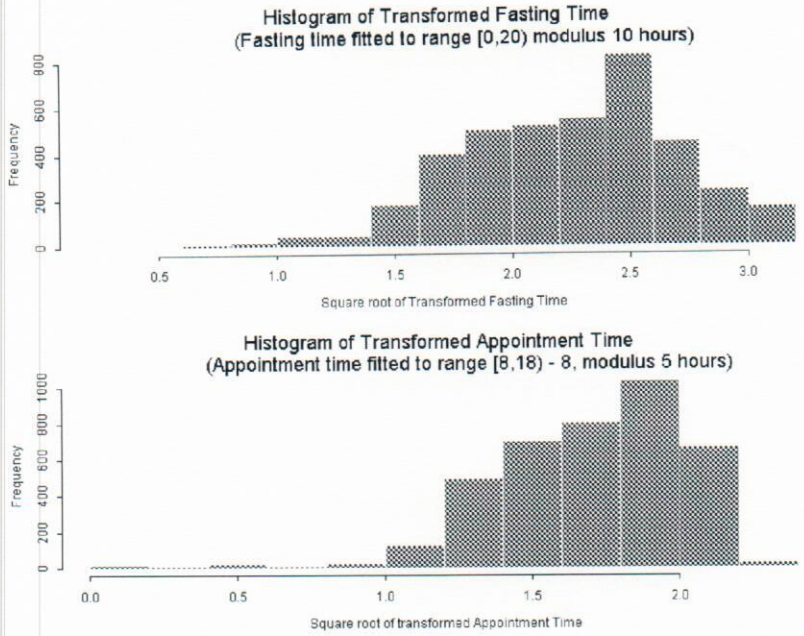
---

[2] Any fasting time that was originally ≥ 20 was changed to 19.99 for the purpose of modulus division.

[3] The new data are just a linear combination of the original data $\xi_{\text{original}} = \xi_{\text{new}} + 10\delta$, where δ equals one or zero, depending on whether ξ_{original} was less than 10 hours.

[4] The new data are just a linear combination of the original data $\tau_{\text{original}} = \tau_{\text{new}} + 8 + 5\gamma$, where γ equals one or zero, depending on whether τ_{original} was less than 13 hours (i.e. before a 1 P.M appointment).

[5] A power transformation method (Box-Cox) could have been used to determine a more suitable power transformation than the square root, but it was decided to keep things as simple as possible from here on.



INSULIN & GLUCOSE ADJUSTMENT

Having transformed the relevant variables, models were fitted for insulin and glucose, for diabetics and non diabetics separately. The main effects of fasting duration (2 independent variables) and time of appointment (2 independent variables) were included along with all 2 way interactions. A backwards elimination procedure was then adopted to select the best model in each case. Based on the coefficients of these models, subjects were then standardised to that of a 6 hour faster with a 1P.M.appointment.

SENSITIVITY ANALYSIS – GLYCOSYLATED HAEMOGLOBIN (HBA1C)

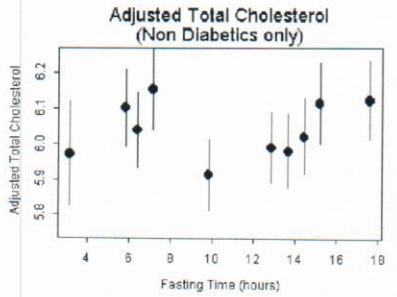
It is possible that the diabetics that failed to fast may in some sense be 'worst case diabetics', and so adjustment of their insulin and glucose levels may actually be adjusting away true differences between the diabetics. To investigate this possibility a sensitivity analysis was carried out for the diabetics that included in the models the variable HBA1C. Backwards elimination of the full model was again carried out for both insulin and glucose.

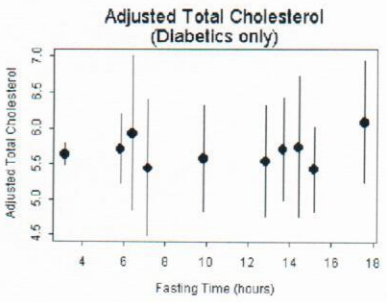
For insulin, the re-fitted model contained the same combination of variables as before (plus HBA1C), and the coefficients were similar in direction and magnitude as in the model where we don't adjust for HBA1C.

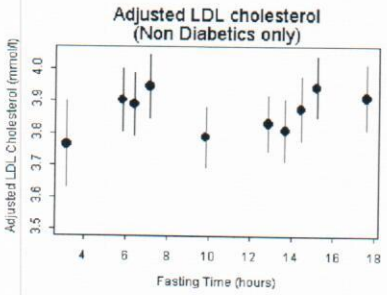
For glucose, the re-fitted model becomes simpler, involving HBA1C and the two main effects of fasting time (transformed continuous variable) and whether or not the appointment was in the morning. HBA1C is included in the adjustment models for the purpose of preserving these differences between diabetics.

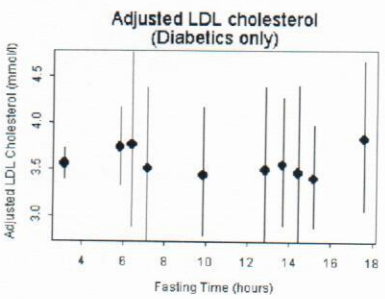
AFTER THE ADJUSTMENT

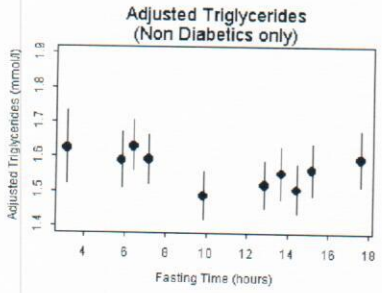
Results of all the adjustments are shown below.

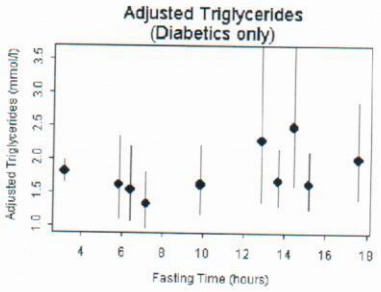


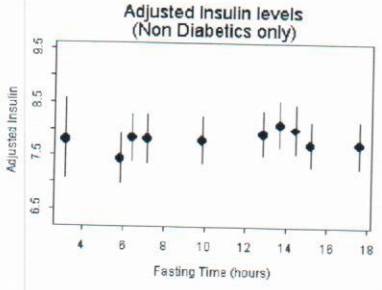


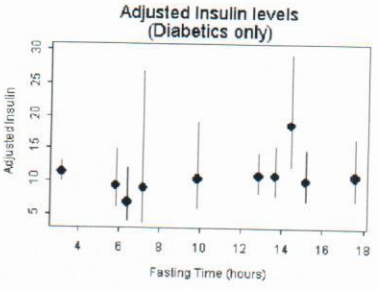


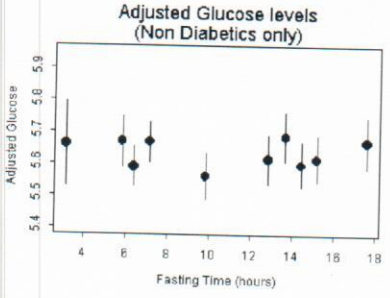


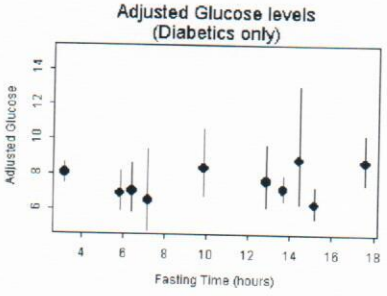












DISCUSSION

For the twenty year follow up of men in the British Regional Heart Study, it was necessary to adjust certain blood measurements to take into account the different fasting times of the subjects. Five measurements were adjusted, total cholesterol, LDL cholesterol, triglycerides, insulin and glucose. Diabetic subjects were adjusted differently to non diabetics for all measures except total cholesterol.

Simple adjustments were carried out for total cholesterol, LDL cholesterol and triglycerides, which only adjusted non fasters (< 6 hours fasting duration) to a level consistent with fasters. This kind of adjustment is straightforward, but makes no distinction between fasting times of non-fasters. Some subjects falling into the category of non fasters would most likely be adjusted by an amount more than that expected if they had truly fasted for 6 hours. Alternatively, some subjects may still have artificially low readings.

For insulin and glucose, it was necessary to make a distinction between afternoon appointments and morning appointments, as it appeared that many subjects may well have miss-reported their true fasting time (potentially due to a drink that they neglected to mention). It is not surprising that such an error would present itself when analysing insulin and glucose, but be hidden from the other measurements. For these two measures then, a more complicated adjustment was performed, including aspects of both fasting time and time of appointment in the adjustment. A sensitivity analysis using the marker of HBA1C yielded similar results in terms of coefficients. HBA1C was included in the final models for insulin and glucose, partly since the model for glucose was simpler with HBA1C in it, than it was without.

No account of town of residence was used in these analyses. It is assumed that fasting status in independent of town of residence.

```
# ============================
# FASTING ADJUSTMENT PROGRAM
# ============================

# In this program we adjust for the fasting duration of each subject.
# For total cholesterol, LDL cholesterol and triglycerides, we adjust for
# fasting status only, i.e. we adjust those who didn't fast to a level
# consistent with those who did (whilst preserving differences between diabetics
# and non diabetics).  No adjustment is carried out for HDL chlesterol as difference
s
# between fasting levels are consistent with a chance event.  For insulin and glucos
e
# we adjust based on two factors, fasting duration and time of appointment, both of
# which we consider as continuous variables.  We standardise responses to those of a

# 6 hour faster with a 1pm appointment (the mean appointment time).


import.data(FileName = "C:\\work\\brhs\\fasting adjustment\\output\\Fstm_tod.sd2",
        FileType = "SAS",
        ColNames = "",
        Format = "",
        TargetStartCol = "1",
        DataFrame = "fasting.adjustment.data",
        NameRow = "",
        StartCol = "1",
        EndCol = "END",
        StartRow = "1",
        EndRow = "END",
        Delimiters = ", \t",
        SeparateDelimiters = F,
        PageNumber = "1",
        RowNameCol = "",
        StringsAsFactors = "Auto",
        VLabelAsNumber = F,
        Filter = "",
        OdbcConnection = "",
        OdbcSqlQuery = "")

  fasting.adjustment.data$DIABETES <- factor(fasting.adjustment.data$DIABETES)
  fasting.adjustment.data$DECGRP <- factor(fasting.adjustment.data$DECGRP)

##################################################
########  TOTAL CHOLESTEROL ADJUSTMENT ###########
##################################################

#  WE FOUND THAT FOR TOTAL CHOLESTEROL, NON FASTERS (< 6 HRS) HAD ON AVERAGE
#  LOWER CHOLESTEROL LEVELS THAN FASTERS (>6 HRS), AND THAT DIABETICS
#  HAD LOWER CHOLESTEROL LEVELS THAN NON-DIABETICS.  THE DIFFERENCE BETWEEN
#  FASTERS AND NON-FASTERS DID NOT VARY BY DIABETIC STATUS (NON-SIGNIFICANT
#  INTERACTION)

        chol.model <- lm(CHOL ~ DIABETES + SUC.FAST, data=fasting.adjustment.data, n
a.action=na.omit)
        fasting.adjustment.data$CHOL <- fasting.adjustment.data$CHOL +
                                (fasting.adjustment.data$SUC.FAST==0)*coef(chol.mode
l)[3]

##################################################
########  HDL CHOLESTEROL ADJUSTMENT ###########
##################################################

# NO ADJUSTMENT IS CARRIED OUT FOR HDL CHOLESTEROL.  AS WE CAN SEE FROM THE
# FOLLOWING REGRESSION, NO ADJUSTMENT IS NECESSARY.

        hdl.model <- lm(sqrt(HDL) ~ DIABETES + SUC.FAST, data=fasting.adjustment.dat
a, na.action=na.omit)
        summary(hdl.model)
```

```
################################################
########  LDL CHOLESTEROL ADJUSTMENT ############
################################################

        ## THE ORIGINAL DATA SHOW SIGNIFICANT MAIN EFFECTS DUE TO DIABETIC STATUS AN
D FASTING STATUS
        ## THEY ALSO INDICATE A SIGNIFICANT INTERACTION WHICH IS EVIDENT FROM THE IN
TERACTION PLOTS
        ## CONTAINED IN 'C:\WORK\BRHS\FASTING ADJUSTMENT\GRAPHS\INTERACTION PLOTS 1
& 2'

        ## WE THERFORE ADJUST THE DIABETICS AND NON DIABETICS SEPARATELY BASED ON 2
MODELS

        # STEP 1- ADJUST THE NON-DIABETICS
        # ================================
        ldl.model <- lm(sqrt(LDL) ~ SUC.FAST, data=fasting.adjustment.data, na.actio
n=na.omit,
                                        subset=(fasting.adjustment.data$DIABETES!=1)
)
        summary(ldl.model)
        new.sqrt.ldl <- sqrt(fasting.adjustment.data$LDL[fasting.adjustment.data$DIA
BETES!=1]) +
                                (fasting.adjustment.data$SUC.FAST[fasting.adjustment
.data$DIABETES!=1]==0)*coef(ldl.model)[2]
        fasting.adjustment.data$LDL[fasting.adjustment.data$DIABETES!=1] <- new.sqrt
.ldl^2


        # STEP 2 - ADJUST THE DIABETICS
        # =====================================
        ldl.model3 <- lm(sqrt(LDL) ~ SUC.FAST, data=fasting.adjustment.data, na.acti
on=na.omit,
                                        subset=(fasting.adjustment.data$DIABETES==1)
)
        summary(ldl.model3)
        new.sqrt.ldl <- sqrt(fasting.adjustment.data$LDL[fasting.adjustment.data$DIA
BETES==1]) +
                                (fasting.adjustment.data$SUC.FAST[fasting.adjustment
.data$DIABETES==1]==0)*coef(ldl.model3)[2]
        fasting.adjustment.data$LDL[fasting.adjustment.data$DIABETES==1] <- new.sqrt
.ldl^2



################################################
########  TRIGLYCERIDES ADJUSTMENT ############
################################################

        # SIMILAR SITUATION TO LDL CHOLESTEROL -> ADJUST USING TWO MODELS

        # STEP 1- ADJUST THE NON-DIABETICS
        # ================================
        trig.model <- lm(log(TRIGS) ~ SUC.FAST, data=fasting.adjustment.data,
                                        na.action=na.omit, subset=(fasting.adjustmen
t.data$DIABETES!=1))
        summary(trig.model)
        new.logtrig <- log(fasting.adjustment.data$TRIGS[fasting.adjustment.data$DIA
BETES!=1]) +
                                (fasting.adjustment.data$SUC.FAST[fasting.adjustment
.data$DIABETES!=1]==0)*coef(trig.model)[2]
        fasting.adjustment.data$TRIGS[fasting.adjustment.data$DIABETES!=1] <- exp(ne
w.logtrig)


        # STEP 2 - ADJUST THE DIABETICS
        # =================================
        trig.model3 <- lm(log(TRIGS) ~ SUC.FAST, data=fasting.adjustment.data, na.ac
tion=na.omit,
```

```
                                                subset=(fasting.adjustment.data$DIABETES==1)
 )
        summary(trig.model3)
        new.logtrig <- log(fasting.adjustment.data$TRIGS[fasting.adjustment.data$DIA
 BETES==1]) +
                                    (fasting.adjustment.data$SUC.FAST[fasting.adjustment
 .data$DIABETES==1]==0)*coef(trig.model3)[2]
        fasting.adjustment.data$TRIGS[fasting.adjustment.data$DIABETES==1] <- exp(ne
 w.logtrig)



 ##########################
 ### INSULIN ADJUSTMENT ###
 ##########################

 # Both the fasting time and time of appointment are bi-modal
 # Fasting time reaches a peak at 7 hours, falls towards 10 hours, and then
 # reaches another peak at approximately 15 hours. NB/ 55% of the subjects fasted
 # for at least 10 hours.
 # Time of appointment has two peaks corresponding to the busy periods
 # in the morning and the afternoon.
 # We transform these variables to be normal in the following way:
 # For fasting time we take as the new variable fasting time modulus 10 (NB/ We
 # don't lose the information on who fasted as we keep the variable LONGFAST, defined
  to be 1
 # if the subject fasted for >= 10 hours, and a zero otherwise)
 # For time of appointment we take as the new variable the number of hours since
 # either 8am or 1pm, depending on whether the appointment is in the afternoon or mor
 ning.
 # NB/ We don't lose the information regarding the morning/afternoon appointments, si
 nce
 # we keep the binary varible MORNING.

 par(mfrow=c(2,1))
 hist(fasting.adjustment.data$FAST.TM[fasting.adjustment.data$DIABETES!=1], xlab='Fas
 ting Duration', ylab='Frequency', main='Histogram of Fasting Duration')
 hist(fasting.adjustment.data$TIMEDSX[fasting.adjustment.data$DIABETES!=1], xlab='Tim
 e of Appointment', ylab='Frequency', main='Histogram of Appointment Time')

 past.12hours <- fasting.adjustment.data$FAST.TM >= 20
 fasting.adjustment.data$FAST.TM[past.12hours] <- 19.99
 fasting.adjustment.data$FAST.TM <- fasting.adjustment.data$FAST.TM %% 10
 past.6pm <- fasting.adjustment.data$TIMEDSX >= 18
 fasting.adjustment.data$TIMEDSX[past.6pm] <- 17.99
 fasting.adjustment.data$TIMEDSX <- (fasting.adjustment.data$TIMEDSX - 8) %% 5

 hist(sqrt(fasting.adjustment.data$FAST.TM[fasting.adjustment.data$DIABETES!=1]), xla
 b='Square root of Transformed Fasting Time', ylab='Frequency', main='Histogram of Tr
 ansformed Fasting Time
 (Fasting time fitted to range [0,20) modulus 10 hours)')
 hist(sqrt(fasting.adjustment.data$TIMEDSX[fasting.adjustment.data$DIABETES!=1]), xla
 b='Square root of transformed Appointment Time', ylab='Frequency', main='Histogram o
 f Transformed Appointment Time
 (Appointment time fitted to range [8,18) - 8, modulus 5 hours)')

        # STEP 1- ADJUSTMENT FOR TIME OF APPOINTMENT AND FASTING STATUS - FOR THE NO
 N DIABETICS ONLY
        # ============================================================================
 ================
        ins.model1 <- lm(log(INSULIN) ~ LONGFAST + MORNING + sqrt(FAST.TM) + sqrt(TI
 MEDSX) +        MORNING:sqrt(TIMEDSX)  + LONGFAST:sqrt(FAST.TM)

                                                ,data=fasting.adjustment.data, na.ac
 tion=na.omit,
                                                subset=(fasting.adjustment.data$DIAB
 ETES!=1))
        summary(ins.model1)
```

```
        # ADJUST THE NON-DIABETIC INSULIN LEVELS TO STANDARDISE TO A 6 HOUR FASTER W
ITH A 1PM APPOINTMENT
        # i.e.   LONGFAST=0, MORNING=0, FAST.TM=6, TIMEDSX=0
        new.logins <- log(fasting.adjustment.data$INSULIN[fasting.adjustment.data$DI
ABETES!=1]) +
                                        (0-fasting.adjustment.data$LONGFAST[fasting.
adjustment.data$DIABETES!=1])*coef(ins.model1)[2] +
                                        (0-fasting.adjustment.data$MORNING[fasting.a
djustment.data$DIABETES!=1])*coef(ins.model1)[3] +
                                        (sqrt(6)-sqrt(fasting.adjustment.data$FAST.T
M[fasting.adjustment.data$DIABETES!=1]))*coef(ins.model1)[4] +
                                        (0-sqrt(fasting.adjustment.data$TIMEDSX[fast
ing.adjustment.data$DIABETES!=1]))*coef(ins.model1)[5] +
                                    (0-(fasting.adjustment.data$MORNING*sqrt(fasting.adj
ustment.data$TIMEDSX))[fasting.adjustment.data$DIABETES!=1])*coef(ins.model1)[6] +
                                    (0-(fasting.adjustment.data$LONGFAST*sqrt(fasting.ad
justment.data$FAST.TM))[fasting.adjustment.data$DIABETES!=1])*coef(ins.model1)[7]

        fasting.adjustment.data$INSULIN[fasting.adjustment.data$DIABETES!=1] <- exp(
new.logins)



        # STEP 2 - ADJUSTMENT FOR TIME OF APPOINTMENT AND FASTING STATUS - FOR THE D
IABETICS ONLY
        # ==========================================================================
=============

        # The non-fasting diabetics may differ from the fasting diabetics as 'worst
case' diabetics.
        # To account for this possibility, we use models adjusting for HBA1C of the
diabetics.
        # We do this for Insulin and also for glucose

        hist(fasting.adjustment.data$HBA1C[fasting.adjustment.data$DIABETES==1], mai
n='HBA1C
        Diabetics only', xlab='HBA1C', ylab='Frequency')
        hist(log(fasting.adjustment.data$HBA1C[fasting.adjustment.data$DIABETES==1])
, main='HBA1C (Log Scale)
        Diabetics only', xlab='HBA1C (Log Scale)', ylab='Frequency')


        ins.model3 <- lm(log(INSULIN) ~ log(HBA1C) + LONGFAST + MORNING + sqrt(FAST.
TM)+ LONGFAST:MORNING + MORNING:sqrt(FAST.TM)
                                                , data=fasting.adjustment.data, na.a
ction=na.omit,
                                                subset=(fasting.adjustment.data$DIAB
ETES==1))
        summary(ins.model3)

        # ADJUST THE NON-DIABETIC INSULIN LEVELS TO STANDARDISE TO A 6 HOUR FASTER W
ITH A 1PM APPOINTMENT
        # i.e.   LONGFAST=0, MORNING=0, FAST.TM=6, TIMEDSX=0
        # THE MEAN FASTING TIME FOR THE DIABETICS WAS 6.3 HOURS

        new.logins <- log(fasting.adjustment.data$INSULIN[fasting.adjustment.data$DI
ABETES==1]) +
                                        (0-fasting.adjustment.data$LONGFAST[fasting.
adjustment.data$DIABETES==1])*coef(ins.model3)[3] +
                                        (0-fasting.adjustment.data$MORNING[fasting.a
djustment.data$DIABETES==1])*coef(ins.model3)[4] +
                                        (sqrt(6)-sqrt(fasting.adjustment.data$FAST.T
M[fasting.adjustment.data$DIABETES==1]))*coef(ins.model3)[5] +
                                    (0-(fasting.adjustment.data$MORNING*fasting.adjustme
nt.data$LONGFAST)[fasting.adjustment.data$DIABETES==1])*coef(ins.model3)[6] +
                                    (0-(fasting.adjustment.data$MORNING*sqrt(fasting.adj
ustment.data$FAST.TM))[fasting.adjustment.data$DIABETES==1])*coef(ins.model3)[7]

        fasting.adjustment.data$INSULIN[fasting.adjustment.data$DIABETES==1] <- exp(
```

```
new.logins)



##########################
### GLUCOSE ADJUSTMENT ###
##########################


        # STEP 1- ADJUSTMENT FOR TIME OF APPOINTMENT AND FASTING STATUS - FOR THE NO
N DIABETICS ONLY
        # ================================================================================
================

        glu.model1 <- lm(log(log(GLU)) ~ LONGFAST + MORNING + sqrt(FAST.TM) + sqrt(T
IMEDSX) + MORNING:sqrt(FAST.TM)+MORNING:sqrt(TIMEDSX)
                                                ,data=fasting.adjustment.data, na.ac

tion=na.omit,
                                                subset=(fasting.adjustment.data$DIAB

ETES!=1))
        summary(glu.model1)

        # ADJUST THE NON-DIABETIC GLUOSE LEVELS TO STANDARDISE TO A 6 HOUR FASTER WI
TH A 1PM APPOINTMENT
        # i.e.   LONGFAST=0, MORNING=0, FAST.TM=6, TIMEDSX=0
        new.glu <- log(log(fasting.adjustment.data$GLU[fasting.adjustment.data$DIABE
TES!=1])) +
                                        (0-fasting.adjustment.data$LONGFAST[fasting.

adjustment.data$DIABETES!=1])*coef(glu.model1)[2] +
                                        (0-fasting.adjustment.data$MORNING[fasting.a

djustment.data$DIABETES!=1])*coef(glu.model1)[3] +
                                        (sqrt(6)-sqrt(fasting.adjustment.data$FAST.T

M[fasting.adjustment.data$DIABETES!=1]))*coef(glu.model1)[4] +
                                        (0-sqrt(fasting.adjustment.data$TIMEDSX[fast

ing.adjustment.data$DIABETES!=1]))*coef(glu.model1)[5] +
                                (0-(fasting.adjustment.data$MORNING*sqrt(fasting.adj

ustment.data$FAST.TM))[fasting.adjustment.data$DIABETES!=1])*coef(glu.model1)[6] +
                                (0-(fasting.adjustment.data$MORNING*sqrt(fasting.adj

ustment.data$TIMEDSX))[fasting.adjustment.data$DIABETES!=1])*coef(glu.model1)[7]

        fasting.adjustment.data$GLU[fasting.adjustment.data$DIABETES!=1] <- exp(exp(

new.glu))


        # STEP 2 - ADJUSTMENT FOR TIME OF APPOINTMENT AND FASTING STATUS - FOR THE D
IABETICS ONLY
        # ================================================================================
=============

        glu.model3 <- lm(log(log(GLU)) ~ log(HBA1C) + MORNING + sqrt(FAST.TM)
                                                ,data=fasting.adjustment.data, na.action=na.

omit,
                                                subset=(fasting.adjustment.data$DIAB

ETES==1))
        summary(glu.model3)

        # ADJUST THE DIABETIC GLUOSE LEVELS TO STANDARDISE TO A 6 HOUR FASTER WITH A
 1PM APPOINTMENT
        # i.e.   MORNING=0, FAST.TM=6
        new.glu <- log(log(fasting.adjustment.data$GLU[fasting.adjustment.data$DIABE
TES==1])) +
                                        (0-fasting.adjustment.data$MORNING[fasting.a

djustment.data$DIABETES==1])*coef(glu.model3)[3] +
                                        (sqrt(6)-sqrt(fasting.adjustment.data$FAST.T

M[fasting.adjustment.data$DIABETES==1]))*coef(glu.model3)[4]

        fasting.adjustment.data$GLU[fasting.adjustment.data$DIABETES==1] <- exp(exp(

new.glu))
```

```
#### CHECK THAT ALL THE ADJUSTMENTS HAVE DONE THEIR JOB !

# TOTAL CHOLESTEROL
        chol.model <- lm(CHOL ~ DIABETES + SUC.FAST, data=fasting.adjustment.data, n
a.action=na.omit)
        summary(chol.model)

# LDL CHOLESTEROL
        ldl.model2 <- lm(sqrt(LDL) ~ SUC.FAST, data=fasting.adjustment.data, na.acti
on=na.omit,
                                        subset=(fasting.adjustment.data$DIABETES!=1)
)
        summary(ldl.model2)
        ldl.model4 <- lm(sqrt(LDL) ~ SUC.FAST, data=fasting.adjustment.data, na.acti
on=na.omit,
                                        subset=(fasting.adjustment.data$DIABETES==1)
)
        summary(ldl.model4)

# TRIGLYCERIDES
        trig.model2 <- lm(log(TRIGS) ~ SUC.FAST, data=fasting.adjustment.data,
                                        na.action=na.omit, subset=(fasting.adjustmen
t.data$DIABETES!=1))
        summary(trig.model2)
        trig.model4 <- lm(log(TRIGS) ~ SUC.FAST, data=fasting.adjustment.data, na.ac
tion=na.omit,
                                        subset=(fasting.adjustment.data$DIABETES==1)
)
        summary(trig.model4)

# INSULIN
        ins.model2 <- lm(log(INSULIN) ~ LONGFAST + MORNING + sqrt(FAST.TM) + sqrt(TI
MEDSX) +        MORNING:sqrt(TIMEDSX)  + LONGFAST:sqrt(FAST.TM)
                                                , data=fasting.adjustment.data, na.a
ction=na.omit,
                                                subset=(fasting.adjustment.data$DIAB
ETES!=1))
        summary(ins.model2)
        ins.model4 <- lm(log(INSULIN) ~ log(HBA1C) + LONGFAST + MORNING + sqrt(FAST.
TM) + LONGFAST:MORNING + MORNING:sqrt(FAST.TM)
                                                , data=fasting.adjustment.data, na.a
ction=na.omit,
                                                subset=(fasting.adjustment.data$DIAB
ETES==1))
        summary(ins.model4)

# GLUCOSE
        glu.model2 <- lm(log(log(GLU)) ~ LONGFAST + MORNING + sqrt(FAST.TM) + sqrt(T
IMEDSX) + MORNING:sqrt(FAST.TM)+MORNING:sqrt(TIMEDSX)
                                                ,data=fasting.adjustment.data, na.ac
tion=na.omit,
                                                subset=(fasting.adjustment.data$DIAB
ETES!=1))
        summary(glu.model2)
        glu.model4 <- lm(log(log(GLU)) ~ log(HBA1C)+ MORNING + sqrt(FAST.TM)
                                        ,data=fasting.adjustment.data, na.action=na.
omit,
                                                subset=(fasting.adjustment.data$DIAB
ETES==1))
        summary(glu.model4)


ADJUSTED.DATA <- data.frame(SERIAL=fasting.adjustment.data$SERIAL,
                                                                NEWCHOL=fasting.adj
ustment.data$CHOL,
                                                                NEWLDL=fasting.adju
```

```
stment.data$LDL,
                                                        NEWTRIG=fasting.adj
ustment.data$TRIGS,
                                                        NEWINS=fasting.adju
stment.data$INSULIN,
                                                        NEWGLU=fasting.adju
stment.data$GLU
                                                       )

export.data(DataSet = "ADJUSTED.DATA",
        Columns = "ALL",
        Rows = "ALL",
        Delimiter = ",",
        ColumnNames = T,
        RowNames = T,
        Quotes = T,
        LineLength = "",
        FileName = "C:\\work\\brhs\\data\\data2000\\FAST_ADJ.sd2",
        FileType = "SAS",
        OdbcConnection = "",
        OdbcTable = "",
        FormatString = "")
```

```
        run;


*============================*;
*= Median of decile groups =*;
*============================*;
proc sort; by decgrp; run;
proc univariate data=fast noprint;
        var fast_tm;
        by decgrp;
        where decgrp ne .;
        output out=meds median=medtime;
        run;


*================*;
*= Non Diabetic =*;
*================*;
proc univariate data=fast noprint;
        var chol sqthdl sqtldl logtrigs logins sqtfib logglu sqhba1c;
        by decgrp;
        where decgrp ne . and diabetes ne 1;
        output out=deciles mean = chol sqthdl sqtldl logtrigs logins sqtfib logglu
sqHBA1C
                                  stdmean = sechol sehdl seldl setrigs seinsul sefib seglu
 seHBA1C
                                  n = nchol nhdl nldl ntrigs ninsulin nfib nglu  nHBA1C;
        run;

data nondiab;
        merge meds deciles;
        by decgrp;
        chol=chol;
        hdl=sqthdl ** 2;
        ldl=sqtldl ** 2;
        trigs=exp(logtrigs);
        insulin=exp(logins2);
        fib=sqtfib ** 2;
        glu=exp(exp(logglu));
        HBA1C=sqHBA1C ** 2;

        up_chol = chol + (tinv(0.975, nchol-1)*sechol);
        up_hdl = (sqthdl + (tinv(0.975, nhdl-1)*sehdl)) ** 2;
        up_ldl = (sqtldl + (tinv(0.975, nldl-1)*seldl)) ** 2;
        up_trigs = exp(logtrigs + (tinv(0.975, ntrigs-1)*setrigs));
        up_ins = exp((logins + (tinv(0.975, ninsulin-1)*seinsul)));
        up_fib = (sqtfib + (tinv(0.975, nfib-1)*sefib)) ** 2;
        up_glu = exp(exp(logglu + (tinv(0.975, nglu-1)*seglu)));
        up_HBA1C = (sqHBA1C + (tinv(0.975, nHBA1C-1)*seHBA1C)) ** 2;

        lo_chol = chol - (tinv(0.975, nchol-1)*sechol);
        lo_hdl = (sqthdl - (tinv(0.975, nhdl-1)*sehdl)) ** 2;
        lo_ldl = (sqtldl - (tinv(0.975, nldl-1)*seldl)) ** 2;
        lo_trigs = exp(logtrigs - (tinv(0.975, ntrigs-1)*setrigs));
        lo_ins = exp((logins - (tinv(0.975, ninsulin-1)*seinsul)));
        lo_fib = (sqtfib - (tinv(0.975, nfib-1)*sefib)) ** 2;
        lo_glu = exp(exp(logglu - (tinv(0.975, nglu-1)*seglu)));
        lo_HBA1C = (sqHBA1C - (tinv(0.975, nHBA1C-1)*seHBA1C)) ** 2;

        keep medtime chol hdl ldl trigs insulin fib glu HBA1C up_chol up_hdl up_ldl
up_trigs
             up_ins up_fib up_glu up_HBA1C lo_chol lo_hdl lo_ldl lo_trigs lo_ins lo_
fib lo_glu lo_HBA1C;
        run;


*=============*;
*= Diabetic =*;
*=============*;
```

```
proc univariate data=fast noprint;
        var chol sqthdl sqtldl logtrigs logins sqtfib logglu sqHBA1C;
        by decgrp;
        where decgrp ne . and diabetes=1;
        output out=deciles mean = chol sqthdl sqtldl logtrigs logins sqtfib logglu s
qHBA1C
                           stdmean = sechol sehdl seldl setrigs seinsul sefib seglu
seHBA1C
                           n = nchol nhdl nldl ntrigs ninsulin nfib nglu nHBA1C;
        run;
data diabetic;
        merge meds deciles;
        by decgrp;
        chol=chol;
        hdl=sqthdl ** 2;
        ldl=sqtldl ** 2;
        trigs=exp(logtrigs);
        insulin=exp(logins);
        fib=sqtfib ** 2;
        glu=exp(exp(logglu));
        HBA1C=sqHBA1C ** 2;

        up_chol = chol + (tinv(0.975, nchol-1)*sechol);
        up_hdl = (sqthdl + (tinv(0.975, nhdl-1)*sehdl)) ** 2;
        up_ldl = (sqtldl + (tinv(0.975, nldl-1)*seldl)) ** 2;
        up_trigs = exp(logtrigs + (tinv(0.975, ntrigs-1)*setrigs));
        up_ins = exp((logins + (tinv(0.975, ninsulin-1)*seinsul)));
        up_fib = (sqtfib + (tinv(0.975, nfib-1)*sefib)) ** 2;
        up_glu = exp(exp(logglu + (tinv(0.975, nglu-1)*seglu)));
        up_HBA1C = (sqHBA1C + (tinv(0.975, nHBA1C-1)*seHBA1C)) ** 2;

        lo_chol = chol - (tinv(0.975, nchol-1)*sechol);
        lo_hdl = (sqthdl - (tinv(0.975, nhdl-1)*sehdl)) ** 2;
        lo_ldl = (sqtldl - (tinv(0.975, nldl-1)*seldl)) ** 2;
        lo_trigs = exp(logtrigs - (tinv(0.975, ntrigs-1)*setrigs));
        lo_ins = exp((logins - (tinv(0.975, ninsulin-1)*seinsul)));
        lo_fib = (sqtfib - (tinv(0.975, nfib-1)*sefib)) ** 2;
        lo_glu = exp(exp(logglu - (tinv(0.975, nglu-1)*seglu)));
        lo_HBA1C = (sqHBA1C - (tinv(0.975, nHBA1C-1)*seHBA1C)) ** 2;

        keep medtime chol hdl ldl trigs insulin fib glu HBA1C up_chol up_hdl up_ldl
up_trigs
             up_ins up_fib up_glu up_HBA1C lo_chol lo_hdl lo_ldl lo_trigs lo_ins lo_
fib lo_glu lo_HBA1C;

        run;


*============================================*;
*= Write these data sets to external files =*;
*============================================*;

%macro write(data, mean, lower, upper, out);
        data &out;
                set &data;
                mean=&mean;
                lower=&lower;
                upper=&upper;
                keep medtime mean lower upper;
                run;
%mend;

%write(nondiab, chol, lo_chol, up_chol, lib1.chol_ND);
%write(nondiab, hdl, lo_hdl, up_hdl, lib1.hdl_ND);
%write(nondiab, ldl, lo_ldl, up_ldl, lib1.ldl_ND);
%write(nondiab, trigs, lo_trigs, up_trigs, lib1.trigs_ND);
%write(nondiab, insulin, lo_ins, up_ins, lib1.ins_ND);
%write(nondiab, fib, lo_fib, up_fib, lib1.fib_ND);
%write(nondiab, glu, lo_glu, up_glu, lib1.glu_ND);
%write(nondiab, HBA1C, lo_HBA1C, up_HBA1C, lib1.HBA1C_ND);
```

```
%write(diabetic, chol, lo_chol, up_chol, lib1.chol_DB);
%write(diabetic, hdl, lo_hdl, up_hdl, lib1.hdl_DB);
%write(diabetic, ldl, lo_ldl, up_ldl, lib1.ldl_DB);
%write(diabetic, trigs, lo_trigs, up_trigs, lib1.trigs_DB);
%write(diabetic, insulin, lo_ins, up_ins, lib1.ins_DB);
%write(diabetic, fib, lo_fib, up_fib, lib1.fib_DB);
%write(diabetic, glu, lo_glu, up_glu, lib1.glu_DB);
%write(diabetic, HBA1C, lo_HBA1C, up_HBA1C, lib1.HBA1C_DB);


*=======================================*;
*=  Produce data for factor level plots =*;
*=======================================*;
proc sort data=fast; by suc_fast diabetes; run;
proc univariate data=fast noprint;
        var chol hdl ldl trigs insulin fib glu;
        by suc_fast diabetes;
        where diabetes ne . and suc_fast ne .;
        output out=fac mean= chol hdl ldl trigs insulin fib glu;
        run;



*=============================================================*;
*= For the non-diabetics, explore the relationship between  =*;
*= fasting decile group and time of appointment             =*;
*=============================================================*;

proc sort data=fast; by decgrp; run;
proc univariate data=fast plot;
        var timedsx;
        by decgrp;
        where diabetes ne 1;
        run;



data lib1.factplot;
        set fac;
        run;
data lib1.fstm_tod;
        set fast;
        if diabetes=. then diabetes=2;
        if fast_tm ne .;
        if timedsx < 13 then morning=1; else morning=0;
        if fast_tm >= 10 then longfast=1; else longfast=0;
        keep SERIAL timedsx fast_tm decgrp diabetes insulin glu morning chol ldl tri
gs suc_fast hdl
             longfast hba1c;
        run;
```

```
*-------------------------------------------------------------------------*;
*-  The information gained from the blood samples may depend on          -*;
*-  whether or not the subject fasted for the requested 6 hours          -*;
*-  before examination.                                                  -*;
*-                                                                       -*;
*-  We determine here which variables are affected by fasting status     -*;
*-  (note the role of diabetic status and work out what to do about it   -*;
*-------------------------------------------------------------------------*;


libname lib1 'c:\work\brhs\fasting adjustment\output';

options nodate nonumber;

%inc 'c:\work\brhs\sasprg\formatting.sas';
%inc 'c:\work\brhs\sasprg\read in baseline data\towns.sas';
%inc 'c:\work\brhs\sasprg\read in baseline data\ages.sas';
%inc 'c:\work\brhs\sasprg\read in 2000 data\blue questionnaire.sas';
%inc 'c:\work\brhs\sasprg\read in 2000 data\data sheets.sas';
%inc 'c:\work\brhs\sasprg\read in 2000 data\biochemical data.sas';
%inc 'c:\work\brhs\sasprg\read in 2000 data\haematological data.sas';
%inc 'c:\work\brhs\sasprg\read in 2000 data\plasma.sas';
%inc 'c:\work\brhs\sasprg\read in 2000 data\insulin.sas';


data fast (rename=(n51=diabetes));
        merge bluall (in=flag1) alldsx towns ages allhea allbio plasma insulin;
        by serial;
        if flag1;

        *-- Height --*;
        if readinad ne . then height=.;

        *-- BMI --*;
        BMI = (10000 * actweigh) / (height * height);

        *---  Sort out the fasting times --*;
        timedsx = time + (time1/60);
        if timedsx=. then timedsx=time;
        last_ate = n200h + (n200m/60);
        if last_ate=. then last_ate=n200h;

        *-- After checking the blue questionnaire we change the following --*;
        *=======================================*;
        if serial=241534 then timedsx=16 + (50/60);
        *=======================================*;
        if (last_ate>timedsx and last_ate ne . and timedsx ne .)
                then fast_tm = timedsx + (24-last_ate);
        if (last_ate<=timedsx and last_ate ne . and timedsx ne .)
                then fast_tm = timedsx - last_ate;

        *-- Andy and myself agreed to set time of the last meal to 9pm --*;
        *-- if they ate yesterday but gave no time                     --*;
        *-- An ASCII file of serial number and fasting time was        --*;
        *-- produced and circulated to everyone in the BRHS            --*;

        if n200Y=1 and last_ate=. then fast_tm=timedsx+3;
        if n200y=1 and (last_ate<=timedsx and last_ate ne . and timedsx ne .)
                then fast_tm = 24 + timedsx - last_ate;
        if last_ate=0 and n200y=1 then fast_tm=timedsx-last_ate;

        if fast_tm >=6 then suc_fast=1; else suc_fast=0;
        if fast_tm=. then suc_fast=.;

        format town town. n51 yesnof.;

run;

proc sort; by serial; run;
```

```
data _null_;
        set fast;
        file 'c:\work\brhs\data\data2000\fastingtimes.dat';
        put @ 1 serial @ 20 fast_tm;
        run;



*=========================;
*=  fasting by diabetes =*;
*=========================;

proc freq data=fast;
        tables suc_fast;
        where suc_fast ne .;
        run;
proc freq data=fast;
        tables diabetes;
        where diabetes ne .;
        run;
proc freq data=fast;
        tables suc_fast * diabetes / nopercent;
        where suc_fast ne . and diabetes ne .;
        run;

*=============================================================================*;
*=  Is there a fasting  / non-fasting difference re. blood variables? =*;
*=============================================================================*;

data fast;
        set fast;
        sqtfib = sqrt(fib);
        sqthdl = sqrt(hdl);
        sqtldl = sqrt(ldl);
        logins = log(insulin);
        logtrigs = log(trigs);
        logglu=log(log(glu));
        sqhba1c=sqrt(hba1c);
        label sqtfib='Square root of fibrinogen'
              sqthdl='Square root of HDL cholesterol'
              sqtldl='Square root of LDL cholesterol'
              logins='Log insulin'
              logtrigs='Log of triglycerides'
              logglu='Log Glucose'
              ;
        run;

*=============================================*;
*-- Do a Friedman Test in S-Plus for glucose --*;
*=============================================*;
data lib1.friedman;
        set fast;
        if suc_fast ne . and diabetes ne .;
        keep glu suc_fast diabetes;
        run;


*============================*;
*-- Do ANOVAs for the rest --*;
*============================*;
%macro doanova1(var, title);
 proc glm data=fast;
       title1 '2 way ANOVA - '  &title;
       title2 'Null hyp is that fasting has no effect';
       class suc_fast diabetes;
       model &var=suc_fast diabetes suc_fast*diabetes;
       means suc_fast diabetes;
       run;
%mend;
```

```
%macro doanova2(var, title);
 proc glm data=fast;
       title1 '2 way ANOVA - '  &title;
       title2 'Null hyp is that fasting has no effect';
       class suc_fast diabetes;
       model &var=suc_fast diabetes;
       means suc_fast diabetes;
       run;
%mend;


%doanova2(chol, 'Total Cholesterol');
%doanova2(sqthdl, 'HDL Cholesterol');
%doanova1(sqtldl, 'LDL cholesterol');
%doanova1(logtrigs, 'Triglycerides');
%doanova1(sqtfib, 'Fibrinogen');

 proc glm data=fast;
       title1 '2 way ANOVA - Fibrinogen (Non Diabetics)';
       title2 'Null hyp is that fasting has no effect';
       class suc_fast;
       model sqtfib=suc_fast;
       means suc_fast;
       where diabetes ne 1;
       run;
 proc glm data=fast;
       title1 '2 way ANOVA - Fibrinogen (Diabetics)';
       title2 'Null hyp is that fasting has no effect';
       class suc_fast;
       model sqtfib=suc_fast;
       means suc_fast;
       where diabetes = 1;
       run;

%doanova1(logins, 'Insulin');
%doanova1(logglu, 'Glucose');


*================================================================;
*================================================================;

* THEN SPLIT FASTING TIMES INTO DECILES AND CATEGORIZE SUBJECTS ;
* AND LOOK AT MEAN (95% CI) LEVELS OF EACH VARIABLE --> SEARCH  ;
* FOR A POSSIBLE THRESHOLD RELATIONSHIP !!!!!                   ;

*================================================================;
*================================================================;


*=========================*;
*= Classify into deciles =*;
*=========================*;
proc univariate data=fast noprint;
       var fast_tm;
       output out=deciles pctlpre=p pctlpts=10 20 30 40 50 60 70 80 90;
       run;

data fast;
       set fast;
       if .<fast_tm<=4.98333 then decgrp=1;
       if 4.98333<fast_tm<=6.15 then decgrp=2;
       if 6.15<fast_tm<=6.725 then decgrp=3;
       if 6.725<fast_tm<=7.88333 then decgrp=4;
       if 7.88333<fast_tm<=12.4333 then decgrp=5;
       if 12.4333<fast_tm<=13.25 then decgrp=6;
       if 13.25<fast_tm<=14 then decgrp=7;
       if 14<fast_tm<=14.75 then decgrp=8;
       if 14.75<fast_tm<=15.7417 then decgrp=9;
       if 15.7417<fast_tm then decgrp=10;
```

Original Article

Biochemical measures in a population-based study: effect of fasting duration and time of day

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Abstract

Background Epidemiological studies generally aim to make simple but unbiased measurements of individuals. For this reason measurements of metabolic variables (including blood lipids, glucose and insulin) are usually carried out after a period of fasting. Few studies have examined the extent to which the use of a defined protocol for fasting in epidemiological studies abolishes the influence of fasting duration and time of day on biochemical measurements.

Methods Cross-sectional survey of British Regional Heart Study participants (4252 men aged 60–79 years), in which men without diabetes were asked to provide a blood sample after fasting for at least 6 h. Serum total, HDL and LDL cholesterol, triglyceride and insulin, and plasma glucose concentrations were measured between 08:00 h and 18:00 h.

Results Non-fasting men had lower mean LDL cholesterol and higher glucose, insulin and triglyceride levels than fasting men; these differences were more marked among diabetics. Among fasting men without diabetes, insulin and glucose levels were strongly related to time of day, falling gradually throughout the morning and remaining stable in the afternoon. Because of these relationships and the dependence of fasting duration on time of day, insulin and glucose displayed a periodic relation with fasting duration above 6 h. These associations were largely abolished by adjustment for time of day; associations with time of day were unaffected by adjustment for fasting duration. Triglyceride concentrations fell with increasing fasting duration. This relationship was also mediated through a gradual increase in triglyceride levels throughout the day. Adjustments to compensate for these variations are described.

Discussion Even after fasting, biochemical measurements may still differ in relation to fasting duration and time of day. In epidemiological studies, it is important to standardize both the period of fasting and the time of day as much as possible, and make adjustments where necessary.

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Introduction

It is well recognized that circulating concentrations of metabolic variables (triglyceride, glucose and insulin in particular) vary strongly depending on the timing of the last meal.[1] In epidemiological studies in which blood samples are taken, subjects are generally asked to fast for a period of time before their examination, in an attempt to obtain unbiased measurements of metabolic parameters in individuals, and to facilitate the use of standardized diagnostic criteria to assess the prevalence of conditions – for example, the 1998 World Health Organization (WHO) criteria for diagnosis and classification of diabetes mellitus.[2] Examples of epidemiological studies using fasting measurements include the Caerphilly Heart Disease Study,[3] Speedwell Heart Disease Study[4] and the Whitehall II study,[5] in which subjects were asked to fast overnight for a minimum of 8 h. In the Whitehall I study, subjects were also asked to fast overnight, but were given a

glucose preparation equivalent to an oral load of 50 g exactly 2 h before the blood sample was taken.[6]

Few reports have examined the extent to which the use of a defined protocol for fasting in epidemiological studies abolishes the influence of fasting duration and time of day on biochemical measurements. In this report we examine the influence of fasting and time of day on measurements of blood lipids (total, LDL and HDL cholesterol, triglyceride), glucose and insulin among 60–79-year-old men who were asked to fast for at least 6 h before providing a blood sample in an epidemiological survey carried out both in mornings and afternoons.

Methods

The British Regional Heart Study is a prospective study of cardiovascular disease in one General Practice in each of 24 British towns, representing all major British regions.[7] Participants were enrolled in 1978–1980 aged 40–59 years and have been followed up for all-cause mortality using the NHS Central Registers and for cardiovascular morbidity through regular 2-yearly review of General Practice records, with fewer than 1% of participants being lost to follow-up.[8]

Between February 1998 and February 2000, all surviving men were invited to attend for a 20-year follow-up assessment at 60–79 years of age. Ethical approval was obtained from all relevant local Research Ethics Committees. The men were asked to fast for a minimum of 6 h, during which they were instructed to drink only water, and to attend for measurement at a pre-specified time between 08:00 and 20:00 h. Men with diabetes who were taking insulin or oral hypoglycaemic treatment were instructed to eat and drink normally. During assessment, men were asked to complete a questionnaire, which documented the last time they had eaten or drunk anything other than water and recorded details of any previous doctor diagnosis of diabetes. All subjects were asked to provide a blood sample, collected using the Sarstedt Monovette system. Plasma (fluoride-oxalate) was collected for measurement of glucose, serum for measurements of lipids and insulin and whole blood (EDTA) for measurement of HbA_{1c}.

Plasma and serum samples were centrifuged, separated and frozen at $-20^{\circ}C$ within 6 h on the day of collection and transferred to central laboratories for analysis; HbA_{1c} samples were transferred for analysis within 36 h. Total, HDL cholesterol and triglyceride were measured using a Hitachi 747 automated analyser (Hitachi, Tokyo, Japan). Total and HDL cholesterol were analysed using the methods of Siedel *et al.*[9] and Sugiuchi *et al.*[10] respectively; LDL-cholesterol values were calculated using the Friedrickson–Friedwald equation. Plasma glucose was measured using a glucose oxidase method[11] using a Falcor 600 automated analyser (A. Menarini Diagnostics, Wokingham, UK). Serum insulin was measured using an enzyme-linked immunosorbent assay (ELISA) which does not cross-react with proinsulin.[12] HbA_{1c} was measured using a Drew Hb Gold HPLC analyser (Drew Scientific Group Plc, Barrow in Furness, UK). Intra- and inter-assay coefficients of variation were $\leqslant 6{\cdot}5\%$ for all concentrations of HDL cholesterol, $\leqslant 3\%$ for total cholesterol and triglycerides and $\leqslant 1{\cdot}5\%$ for plasma glucose. For insulin the inter-assay coefficients of variation were 5·3% for low concentrations (32·7–40·2 pmol/L), 4·3% for medium concentrations (150–183 pmol/L) and 3·0% for high concentrations (210·6–242·4 pmol/L). Intra-assay coefficient variation was $< 5\%$ for all concentrations of insulin. Intra- and inter-assay coefficients of variation for HbA_{1c} were $\leqslant 5\%$ for the usual range of HbA_{1c} values (3·4–6·0% for non-diabetic subjects).

Men were classified as either diabetic or non-diabetic based on their recall of a doctor recorded diagnosis on the study questionnaire. Men who failed to answer this question had their morbidity records checked for any history of diabetes. These men were then allocated to one of the two groups based on this record. A blood sample was taken at the time of examination and the time of sampling recorded.

Statistical methods

A square root transformation was applied to HDL and LDL cholesterol, and a log transform applied to triglycerides, insulin and glucose, to ensure normality (amongst the diabetic and non-diabetic groups). Mean values and standard error bars were calculated on these scales but are transformed back to the original scales for presentation in tables and figures. For example the standard error bars for the log transformed data were calculated by $\exp(\text{mean} \pm \text{SEM})$ where the mean and SEM were calculated on the log scale. These standard error bars are asymmetrical but appear to be symmetrical in the figures due to the large sample size. Fasting duration was calculated for each subject as the difference in hours from the time recorded at screening, when the blood sample was taken, and the time that the subject reported to have last eaten (taken from the study questionnaire). In cases where the subject reported to have last eaten the day before, but gave no actual time, this was recorded as 20:30 h, which was the median time reported by those providing complete information. The fasting time for these patients was then calculated based on this estimate. Two-way analysis of variance (ANOVA) was used to identify differences in the metabolic levels of fasting and non-fasting subjects, whilst preserving differences between diabetics and non-diabetics. An interaction term tested whether the differences due to

Table 1. *Number (percentage) of men (without diabetes) recording various fasting times at different times of the day*

	Reported fasting duration				
	< 6 h	6- 12 h	12- 18 h	> 18 h	Total
Examination time					
Before 10:30 h	24 (4·1%)	38 (6·5%)	513 (87·8%)	9 (1·5%)	584 (100%)
10:30- 13:00 h	153 (9·1%)	115 (6·8%)	1356 (80·5%)	60 (3·6%)	1684 (100%)
13:00- 15:30 h	116 (18·5%)	377 (60·1%)	56 (8·9%)	78 (12·4%)	627 (100%)
After 15:30 h	225 (21·7%)	740 (71·3%)	14 (1·4%)	59 (5·7%)	1038 (100%)

fasting status (<6 h vs $\geqslant 6$ h) differed in magnitude between diabetic groups. Multiple regression was used to relate simultaneously the fasting time and time of appointment with the blood measurements. Adjustments were subsequently carried out only for men without diabetes. Best adjustment models were selected by backwards elimination of a full model containing all two-way interactions. Variables were kept in the model if they met the 0·1 level of significance. To assess the extent of variation in glucose levels due to time of day, a one-way ANOVA was used, based on the ten time intervals formed by the deciles of appointment time.[13] Details of the adjustments used to standardize for fasting duration and time of day are presented in Appendix 1.

Results

Fasting duration and time of day of sampling

Of 4252 men who attended the 20-year screening (76% of those invited), 4220 provided information on fasting duration, of whom 4016 (95%) provided a full blood sample. Of 4064 men with known reported fasting duration and not taking insulin or oral hypoglycaemic treatment, 3475 (86%) claimed to have fasted for at least 6 h before their appointment. Among 156 men taking insulin or oral hypoglycaemic treatment, 34 (24%) still fasted for at least 6 h. A total of 3933 men were classified as subjects without diabetes, 287 as subjects with diabetes.

The inter-relations of fasting durations and time of appointment for men without diabetes are shown in Table 1. Both were bi-modal. Fasting duration peaked at 7 h and 14 h. This was due to men with morning appointments reporting overnight, and hence longer fasts compared with the men whose appointment was in the afternoon. Peaks in appointment times around 11:00 and 16:00 corresponded to the busiest periods of the survey day. Fasting duration was strongly related to time of day of measurement. Over 80% of the men whose appointment was in the morning (before 13:00 h) reported fasting durations of 12 h or longer. This would indicate that they fasted overnight before their appointment as would be expected. Of the men whose appointment was in the afternoon, approximately 65% reported fasting durations between 6 and 12 h, with a considerable number reporting fasting durations of less than 6 h (approximately 20%). Men with morning appointments were significantly more likely to report very long fasting durations (>10 h) than men with afternoon appointments (89% vs 14%, $P<0·0001$), and more likely to succeed in fasting for 6 h than men with afternoon appointments (92% vs 80%, $P<0·0001$). There were no consistent differences in age or body mass index (BMI) of subjects who fasted for different durations, or were measured at different times of day.

Influence of fasting duration and time of day on metabolic variables

The relationship between biochemical measurements and fasting status (whether or not subjects had fasted for 6 h or not) is shown in Table 2 for both subjects with and without diabetes. Men with diabetes had lower total cholesterol, HDL cholesterol and LDL cholesterol, and higher triglycerides, insulin and glucose than men without diabetes (all $P<0·001$). Among all men who had not fasted for 6 h, mean total and LDL cholesterol were lower and mean triglyceride, glucose and insulin higher when compared with fasting men (adjusting for diabetic status). With the exception of total cholesterol the influence of fasting status was more marked among the diabetic group. HDL cholesterol level showed no relation with fasting status.

Because the influence of fasting status appears to differ between the diabetic groups and because the fasting status of subjects with diabetes is likely to be related to the severity of their diabetes, more detailed analyses of fasting duration and time of day are restricted to non-diabetic subjects.

Figure 1 shows the relationship between fasting duration and biochemical measurements, presented separately for men without diabetes measured in the morning and afternoon. It can be seen that men whose appointment was in the morning on the whole did not fast for between 4 and 9 h, whereas men in the afternoon rarely fasted for longer than 10 h, these

Table 2. *Mean (95% confidence interval) biochemical measurements in men with and without diabetes, according to whether they fasted for at least 6 h*

Mean measurement (95% CI)	Non-fasters (< 6 h)	Fasters (⩾ 6 h)	*P value*
Total cholesterol (mmol/L)	(all men - non-fasters vs fasters)		0·03
Diabetics	5·54 (5·39- 5·70)	5·78 (5·56- 5·99)	0·09
Non-diabetics	5·95 (5·86- 6·05)	6·04 (6·00- 6·08)	0·09
HDL cholesterol* (mmol/L)	(all men - non-fasters vs fasters)		0·32
Diabetics	1·15 (1·11- 1·20)	1·21 (1·15- 1·27)	0·18
Non-diabetics	1·30 (1·27- 1·33)	1·31 (1·30- 1·32)	0·56
LDL cholesterol*†† (mmol/L)			
Diabetics	3·15 (3·01- 3·30)	3·61 (3·40- 3·82)	< 0·001
Non-diabetics	3·70 (3·61- 3·78)	3·87 (3·84- 3·90)	< 0·001
Triglycerides**§ (mmol/L)			
Diabetics	2·41 (2·22- 2·61)	1·81 (1·61- 2·02)	< 0·001
Non-diabetics	1·75 (1·67- 1·82)	1·56 (1·54- 1·59)	< 0·001
Insulin†§ (pmol/L)			
Diabetics	152·5 (135·1- 172·2)	65·4 (55·3- 77·4)	< 0·001
Non-diabetics	47·6 (45·2- 50·2)	47·0 (46·1- 48·0)	0·65
Plasma glucose†§ (mmol/L)			
Diabetics	9·68 (9·11- 10·30)	8·12 (7·46- 8·84)	< 0·01
Non-diabetics	5·64 (5·57- 5·72)	5·70 (5·67- 5·73)	0·13

*$P < 0·05$ for test of interaction between fasting status and diabetic status.
**$P < 0·01$ for test of interaction between fasting status and diabetic status.
†$P < 0·001$ for test of interaction between fasting status and diabetic status.
††Means and confidence intervals calculated on the square root scale.
§Means and confidence intervals calculated on the log scale.

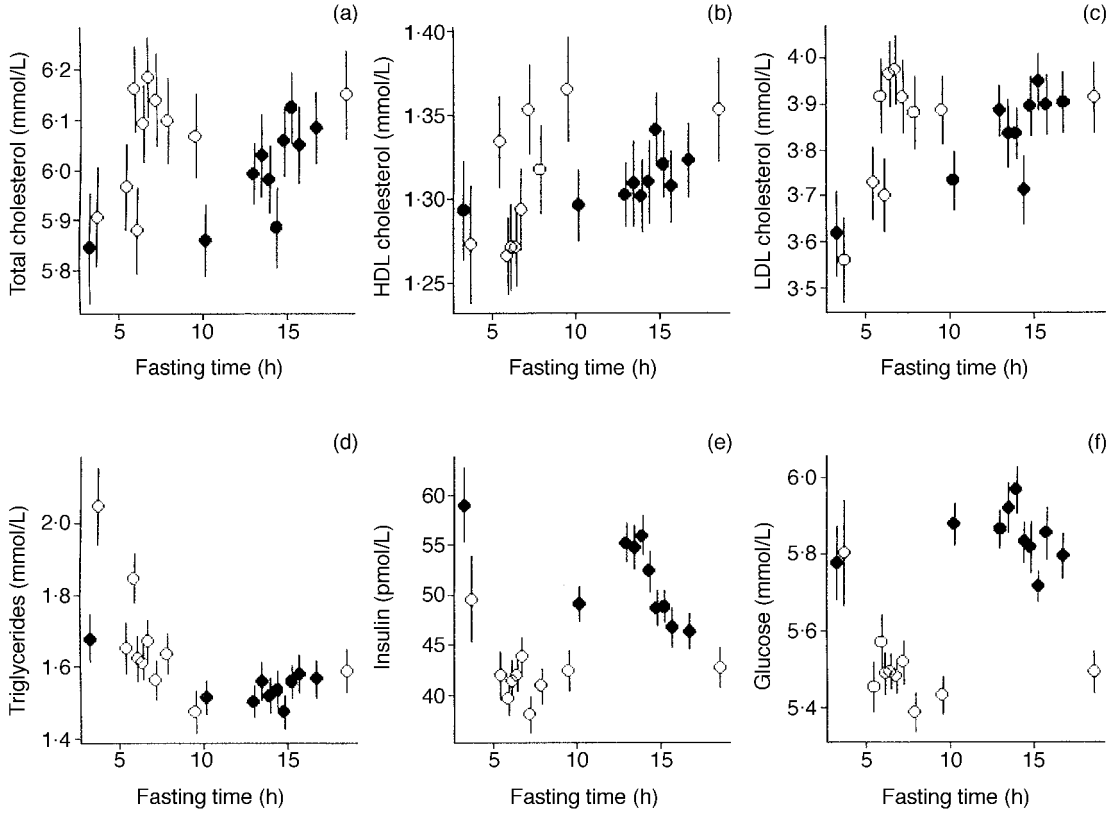

Figure 1. *Biochemical measurements (mean ± standard error of the mean) by fasting duration among men without diabetes. (a) Total cholesterol; (b) HDL cholesterol; (c) LDL cholesterol; (d) triglycerides; (e) insulin; (f) glucose.* ● = *Men whose appointments were in the morning;* ○ = *men with appointments in the afternoon.*

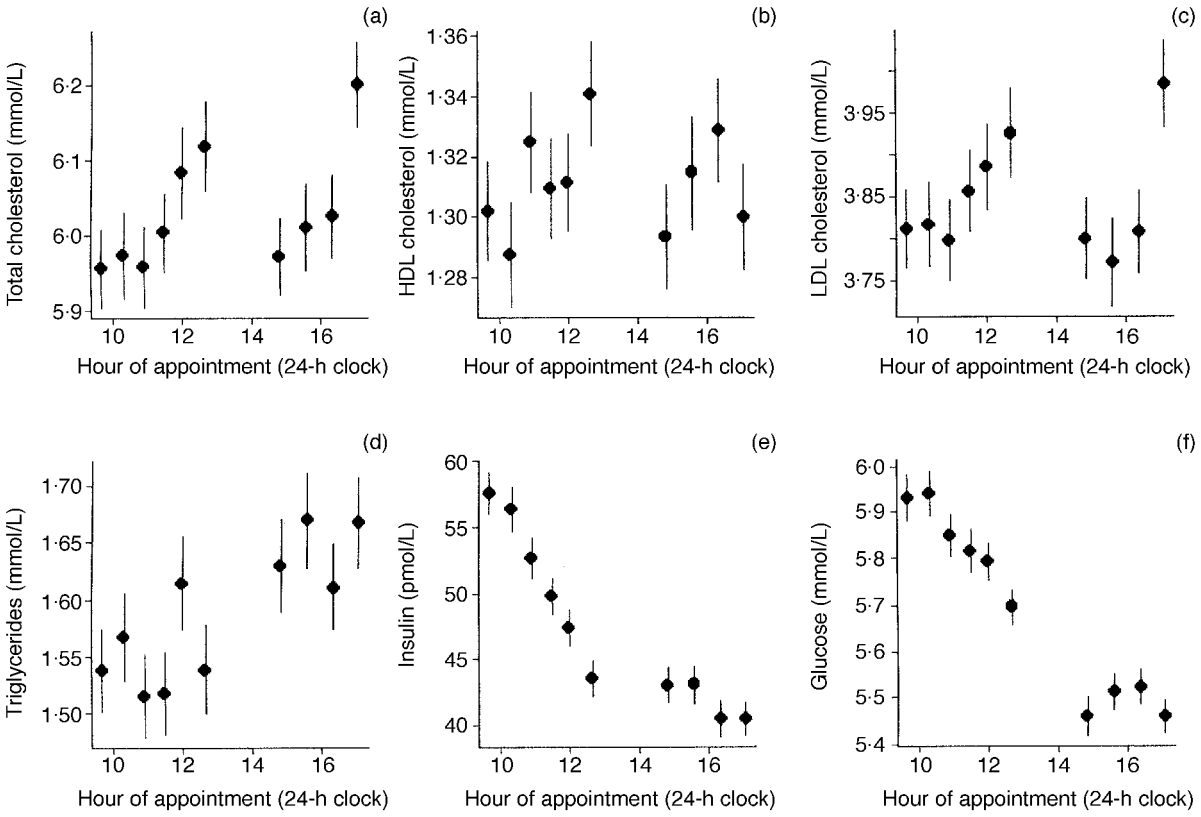

Figure 2. *Biochemical measurements (mean ± standard error of the mean) by time of appointment (deciles) for men without diabetes. (a) Total cholesterol; (b) HDL cholesterol; (c) LDL cholesterol; (d) triglycerides; (e) insulin; (f) glucose.*

times being consistent with normal sleeping patterns. The effects of fasting for fewer than 6 h have already been described. For subjects fasting for more than 6 h, fasting duration was not strongly related to total, LDL or HDL cholesterol; the observed variation being consistent with the role of chance ($P = 0{\cdot}93$, $P = 0{\cdot}44$, $P = 0{\cdot}64$, respectively). In contrast, there were marked variations in triglyceride, insulin and glucose ($P = 0{\cdot}04$, $P < 0{\cdot}001$, $P < 0{\cdot}001$, respectively).

Triglyceride concentrations declined with increasing fasting duration to approximately 10 h, where they reached a plateau. Both insulin and glucose concentrations declined with increasing fasting duration to about 10 h, and then reached a second peak at 10–12 h, from which they declined. This second peak and decline was observed entirely in subjects measured in the morning who had markedly higher insulin and glucose levels than those measured in the afternoon.

Relationships of biochemical measures with time of day are shown in Fig. 2, where the deciles of appointment time have been used to classify the men without diabetes into one of ten groups. Total cholesterol, LDL cholesterol and HDL cholesterol showed no consistent trends with hour of appointment, although total cholesterol did display significant variation.

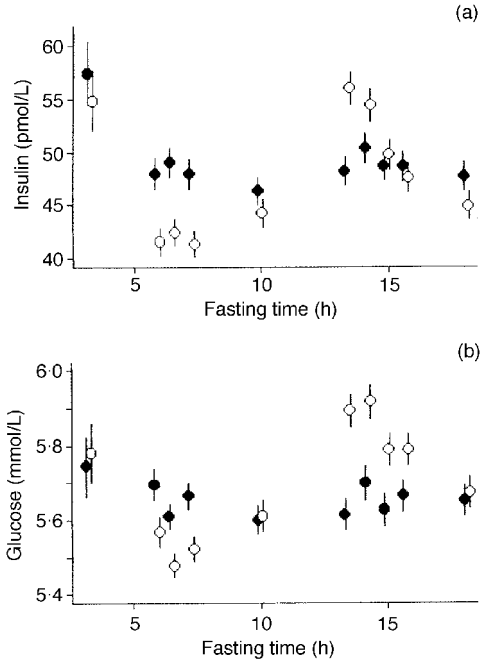

Figure 3. *(a) Serum insulin and (b) plasma glucose levels for men without diabetes (mean ± standard error of the mean). ○ = Unadjusted values; ● = adjusted for time-of-day effects only.*

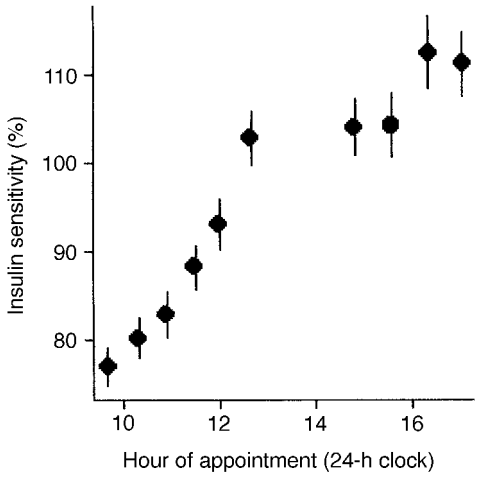

Figure 4. *Insulin sensitivity (mean ± standard error of the mean) by time of day for fasting men without diabetes. Values are relative to a young, lean population (100%).*

Triglycerides rose slowly, but significantly, throughout the day ($P = 0{\cdot}01$). Glucose and insulin concentrations were highest in the morning, and fell progressively throughout the day, levelling off in the afternoon (both $P < 0{\cdot}001$). Adjustment for time of day abolished the relationship between fasting time above 6 h, and insulin and glucose concentrations ($P = 0{\cdot}87$ and $P = 0{\cdot}51$, respectively; *see* Fig. 3). In contrast, time of day associations were unaffected by adjustment for fasting duration, or by adjustment for age, body mass or HbA_{1c} level (morning insulin concentrations were 25% greater than afternoon concentrations before adjusting for these factors and 22% greater after adjusting; these differences for glucose were 7% and 6%, respectively). Exclusion of men whose glucose concentration was 7 mmol/L or more[2] had no effect on the observed relations with fasting time and time of day.

Insulin sensitivity was calculated using the HOMA (homeostasis model assessment) equation for use with specific insulin assays[14] (J Levy, personal communication), for men who successfully fasted for at least 6 h. The relationship between insulin sensitivity and time of day is displayed in Fig. 4 where sensitivity is measured relative to a young lean reference population (who are assigned the value 100%). Insulin sensitivity is lowest in the early morning, and it increases linearly towards the afternoon where it remains constant for the remainder of the day.

Adjustments

Based on the relationships of the six biochemical measurements with fasting duration and time of day, adjustments for these factors were carried out for the men without diabetes. For LDL cholesterol

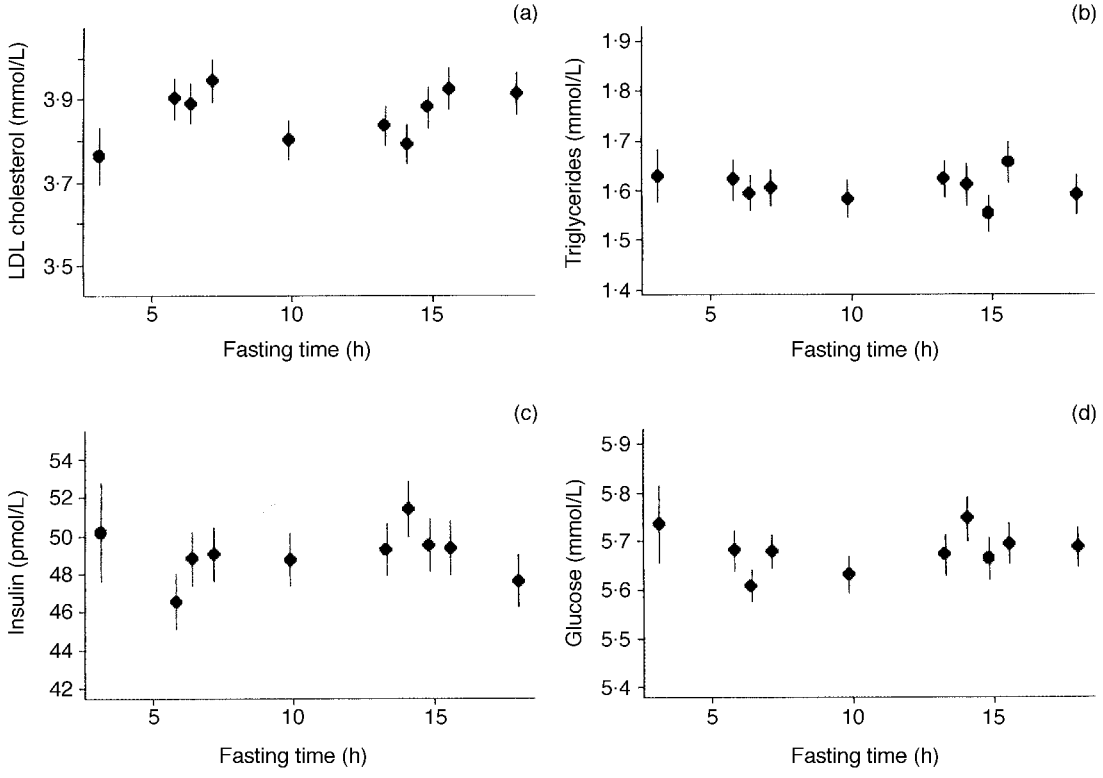

Figure 5. *Biochemical measures (mean ± standard error of the mean) for the men without diabetes after the entire adjustment process. (a) LDL cholesterol; (b) triglycerides; (c) insulin; (d) glucose.*

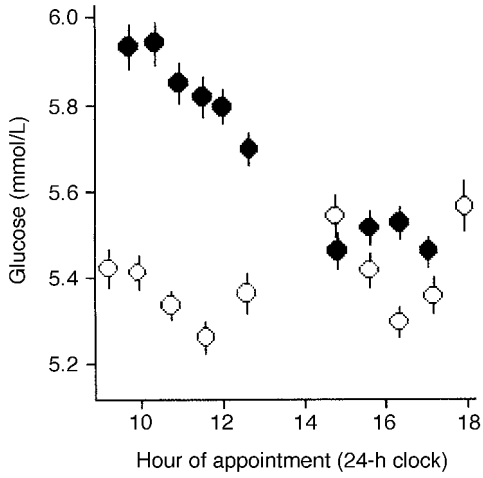

Figure 6. *Non-fasting baseline glucose (○) and fasting 20-year survey glucose levels (●) of a group of 3767 men who had never been diagnosed diabetic (mean ± standard error of the mean).*

concentrations, in which there was little variation above 6 h fasting duration and little diurnal variation, values for non-fasting men were adjusted to those of all fasting men. For triglycerides, insulin and glucose, adjustments were made to standardize measures to those of a man with a fasting duration of 6 h, and an appointment at 13:00 h. Again, adjustments were only applied to the non-diabetic group. Total and HDL cholesterol levels were left unadjusted.

The effects of the adjustments are shown in Fig. 5. For LDL cholesterol, variation between subjects who reported to have fasted successfully was unchanged. The adjusted measurements of triglycerides, insulin and glucose show little variation with fasting duration or time of day because of the greater precision of the adjustments applied in these cases.

Influence of time of day on biochemical measurements: comparison of fasting and non-fasting sampling regimens

At the baseline screening (1978–1980), the men had not been asked to fast before their examination. A comparison of the two instruction regimens, asking men to fast and not asking men to fast, is therefore available using the fasted samples of the 3933 men without diabetes at the 20-year screening with the non-fasted sample taken from the same men at the baseline examination. Figure 6 shows how glucose varied by time of day, both in the baseline sample taken between 1978 and 1980 and the fasted sample taken 20 years later. Although there is marked diurnal variation in the non-fasting sample, with obvious mealtime peaks, the extent of variation is not as marked as in the fasting 20-year samples. The percentage of variance attributable to hour of day was 0·9% in the baseline study and 3% in the 20-year survey – the latter figure rose to 6% if only those subjects claiming to have fasted for 6 h were included.

Discussion

The results of this study suggest that even when a standardized fasting regimen is used, marked differences in metabolic variables can occur, in relation to both fasting duration and, in particular, to time of day. These differences need to be taken into account in analysis. Fasting for fewer than 6 h is associated with lower mean concentrations of LDL cholesterol and higher mean concentrations of triglyceride, insulin and glucose and no effect on total and HDL cholesterol. However, while LDL cholesterol shows little consistent relation with fasting duration above 6 h, triglyceride concentrations show a marked decline with increased fasting duration and insulin and glucose a biphasic decline. Both insulin and glucose show a strong diurnal pattern, with markedly raised concentrations in the early morning. The observed patterns by fasting duration are unchanged by exclusion of the men ($n = 1615$, 38%) for whom exact time of last meal was estimated.

The marked diurnal variations in fasting glucose and insulin concentrations, with particularly high levels in the early morning, are likely to be physiological. They are not explained by differences in the glucose tolerance of individual subjects measured at different times of day, as reflected by their HbA_{1c} levels. Though they could be explained by a systematic tendency to under-report the consumption of food or drink before morning measurement, the absence of a parallel early morning peak in triglyceride concentrations, which would be apparent even after the consumption of a cup of tea or coffee with milk, makes this unlikely. Similar patterns of diurnal variations in fasting glucose and insulin concentrations have been previously described both in population-based studies[15] and in clinical studies of subjects both with and without type 2 diabetes.[16] These variations, which may result from the increase in early morning cortisol concentrations,[17] appear distinct from the diurnal variations in glucose tolerance described, in which glucose tolerance is diminished in the afternoon and evening.[18–21] The diurnal variation in triglyceride concentrations in this study, with a gradual rise in concentrations during the day, corresponds to observations both in the earlier phase of the present study and in earlier reports.[13,21,22] Preliminary findings from the British Women's Heart and Health Study indicate similar observed patterns for women (D Lawlor, personal communication).

Adjustments

Adjustments were developed only for subjects without diabetes. Because the likelihood of men with diabetes fasting was likely to be related to the metabolic severity of their diabetes (men with diabetes were only asked to fast before their examination if they were not on oral hypoglycaemic treatment or insulin), performing adjustments based on such data would potentially provide misleading information. For these reasons it has not been carried out in this analysis.

Of the six variables studied among men without diabetes, adjustments were not necessary for total or HDL cholesterol and only a simple adjustment (fasted/non-fasted) was necessary for LDL cholesterol. The effect of this LDL cholesterol adjustment was small, changing LDL cholesterol ranks by no more than 6% (increase or decrease). For triglyceride, insulin and glucose, a more complex adjustment was used in which men were standardized to that of a 6-h fast and a 13:00 h appointment. This reference group was chosen for two reasons. Firstly, since the men were only asked to fast for 6 h it is preferable to perform adjustments per protocol, and secondly, the blood levels amongst such a reference group are not likely to be too unrealistic for our population. For instance, it would be unreasonable to assume that our population would naturally fast for much more than 6 h at a time, unless examined in the morning. The effect of these adjustments were more marked, changing ranks up to 15–20% for some men. This would be expected for the type of adjustments performed in these cases and is not indicative of a flaw in the methodology.

Implications for blood sampling in epidemiological studies

This study did not compare the fasting and non-fasting metabolic parameters of individuals at one point in time and so does not allow the relative merits of fasting and non-fasting regimens to be assessed directly. Though diurnal variation in glucose measurements were more marked with a fasting regimen, it is not possible to establish whether the individual results were more or less biased using that approach, or indeed what the source of the excess variation was. The results do suggest, however, that for optimal standardization, studies measuring glucose, insulin and triglyceride should, if possible, restrict measurements to a limited period of the day (morning or afternoon) and possibly also define an upper limit to fasting duration as well as a lower limit (e.g. 8–10 h). Where these steps are not possible, it is important to consider the possibility that time of day and fasting duration continue to affect metabolic parameters even under a fasting regimen, and to adjust the data appropriately.

Conclusion

Biochemical measurements from fasted subjects should not necessarily be accepted as unbiased measurements of individuals. Marked variations with fasting duration and time of day may still occur. In the analysis of metabolic measurements in epidemiological studies, it is important to examine the extent of variation by time of day and fasting duration, and to adjust for it as appropriate. Future studies should aim to standardize both the period of fasting and the time of day at which tests are performed.

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Appendix 1

In order to model linearly with fasting duration and time of day the following transformations were carried out.

A dichotomous variable was defined by categorizing fasting time into one of the two half open intervals, [0, 10), or [10, 20). Any fasting time that was originally $\geqslant 20$ was changed to 19·99. The fasting time was then divided modulo 10, creating a continuous variable defined on the half open interval [0, 10). These transformations enable us to write the original data as a linear combination of the two new variables, a binary variable and a uni-modal symmetric continuous variable.

For time of appointment, a similar calculation was performed. All appointments were categorized as either morning or afternoon appointments, where afternoon appointments began at 13:00 h inclusive. A binary variable then defined the time of appointment as belonging to one of the half open intervals [8, 13) or [13, 18), using the 24-h clock notation. By shifting these intervals to the origin and performing a division modulo 5, a continuous variable was defined on the half open interval [0, 5).

The purpose of these transformations was to keep all the information contained in the fasting time and time of appointment, whilst facilitating linear modelling of the variables. The transformed continuous variables were approximately normally distributed on the square root scale.

Comparatively simple regression based adjustments were adopted in cases where biochemical measurements showed a relationship with fasting duration but little or no relationship with time of day. The method of adjustment in this case was to adjust non-fasting levels to an amount consistent with levels from fasting subjects. For measures that showed substantial variation with time of day as well as fasting duration a more sophisticated adjustment was necessary.

For triglycerides, insulin and glucose, adjustments were carried out based on the transformed variables for fasting duration (two independent variables) and time of appointment (two independent variables) together with all two-way interactions. A backwards elimination procedure was adopted to select the optimum model in each case, with factors remaining in the model if they reached the 0·1 level of significance. Based on the coefficients from these models, subjects were standardized to that of a 6-h faster with a 13:00 h appointment.

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