of mortality by district among the cohort.

METHODS

The JACC study was conducted in 45 areas from 19 prefectures throughout Japan; 3 towns in Hokkaido district, 5 towns in Tohoku district, 5 towns in Kanto district, one city, 3 towns and 2 villages in Chubu district, 8 towns and 2 villages Kinki district, one city and one town in Chugoku district, and 4 cities, 9 towns and one village in Kyushu district. A basic cohort population of 127,477 healthy inhabitants (54,032 males and 73,445 females) in the above areas responded to the baseline questionnaire in 1988-1990. Basic cohort members, including 46,465 men and 64,327 women (110,792 in total) aged 40-79 years at entry, were followed up for about 10 years to the end of 1999.

The follow-up survey was conducted using population registries in local municipalities to determine the vital and residential status of the cohort in each area. All the cases that moved out of the study areas were treated as censored cases. Five subjects who were expunged from their residence record by authorities were also treated as censored cases and included in cases that moved out for computing the cohort numbers by follow up status as of end of 1999. All deaths that occurred in the cohort were ascertained by death certificates from local public health centers in the study areas under the authorities' permission from the Director-General of the Prime Minister's Office (Ministry of Public Management, Home Affairs, Post, and Telecommunications). The underlying causes of death were coded according to the International Classification of Diseases and Injuries (ICD) 10th version by verifying computer-stored data in the Ministry of Health, Labour and Welfare with the permission. Those already coded according to the ICD 9th version (from the time of the baseline survey through 1994) and stored in the computer database in the Ministry of Health and Welfare were re-coded in 1999 according to the ICD 10th version (after 1995), using a specifically developed computer program[4] for converting the ICD 9th code to the 10th.

Follow-up condition (alive, dead, or moved) by sex and age group at entry as of the end of 1999 was computed. For those dead, causes of death, especially of cancer deaths, by sex and age group at entry as of the end of 1999 were also computed. Sex-specific standardized mortality ratios (SMRs) were calculated using sex- and age-specific person-years of following-up and sex- and age-specific mortality rates for all Japan in 1988-1999.[5] From a practical reason, the mortality rates in 1989 were used for the follow-up data in 1988-1990, and by the same manner, the rates in 1992, 1995, and 1998 were used for each three years of follow-up. Confidence intervals of SMR were calculated according to chi-square distribution when the observation number was 10 or larger,[6] and according to Poisson distribution when less than 10.[7]

Our entire study design, which comprised singular and collective use of epidemiologic data and biological materials (serum only), was approved in 2000 by the Ethical Board at Nagoya University School of Medicine, where the central secretariat of the JACC study is located.

RESULTS

Table 1 shows the age distribution of the cohort members aged 40-79 years at the time of their enrolment in the study and their follow-up condition as of the end of 1999. Of 46,465 males, 37,750 (81.2%) were alive, 7,238 (15.6%) were dead, and 1,477 (3.2%) had moved out of the study areas. The figures were 57,016 (88.6%), 4,940 (7.7%), and 2,371 (3.7%) among 64,327 females, respectively. The mean follow-up period was about 10 years.

Total cancer deaths accounted for 38.7% (2,798) and 35.0%

Table 1. Age distribution of cohort members at entry and deaths/move-outs as of the end of 1999.

	Age at entry (year)								
	40-44	45-49	50-54	55-59	60-64	65-69	70-74	75-79	Total
					Male				
Total	6,002	5,806	6,322	7,695	8,429	5,518	4,024	2,669	46,465
Alive	5,518	5,331	5,689	6,725	6,917	4,079	2,334	1,157	37,750
(%)	91.9	91.8	90.0	87.4	82.1	73.9	58.0	43.4	81.2
Dead	147	219	441	785	1337	1306	1572	1431	7238
(%)	2.5	3.8	7.0	10.2	15.9	23.7	39.1	53.6	15.6
Moved	337	256	192	185	175	133	118	81	1477
(%)	5.6	4.4	3.0	2.4	2.1	2.4	2.9	3.0	3.2
					Female				
Total	7,557	7,926	9,108	10,816	11,114	8,602	5,557	3,647	64,327
Alive	7,074	7,485	8,565	10,094	10,092	7,364	4,189	2,153	57,016
(%)	93.6	94.4	94.0	93.3	90.8	85.6	75.4	59.0	88.6
Dead	83	136	236	425	713	944	1099	1304	4940
(%)	1.1	1.7	2.6	3.9	6.4	11.0	19.8	35.8	7.7
Moved	400	305	307	297	309	294	269	190	2371
(%)	5.3	3.9	3.4	2.8	2.8	3.4	4.8	5.2	3.7

Table 2. Age distribution of total deaths, all cancer deaths, and site-specific cancer deaths as of the end of 1999.

	Age at entry (year)								
	40-44	45-49	50-54	55-59	60-64	65-69	70-74	75-79	Total
Total deaths	230	355	677	1,210	2,050	2,250	2,671	2,735	12,178
All cancer deaths	95	154	323	578	1,008	850	845	675	4,528
%	41.3	43.4	47.7	47.8	49.2	37.8	31.6	24.7	37.2
Male									
Total deaths	147	219	441	785	1,337	1,306	1,572	1,431	7,238
Cancer deaths	50	88	194	365	677	515	509	400	2,798
%	34.0	40.2	44.0	46.5	50.6	39.4	32.4	28.0	38.7
Lung(C33,C34)	11	11	37	62	174	136	120	93	644
Stomach(C16)	13	19	36	84	130	109	109	82	582
Liver(C22)	5	12	42	62	104	52	56	33	366
Pancreas(C25)	3	5	9	17	35	30	32	30	161
Colon(C18)	4	5	15	14	32	23	27	24	144
Gall bladder/bile duct(C23,C24)	1	4	5	23	32	34	21	20	140
Rectum(C19,C20)	3	8	9	27	18	19	20	19	123
Esophagus(C15)	4	5	12	19	29	18	14	7	108
Prostate(C61)	0	0	1	4	13	20	33	32	103
Malignant lymphoma(C81-C85)	0	3	6	9	18	13	6	8	63
Mouth(C00-C14)	1	1	0	9	17	8	4	9	49
Bladder(C67)	0	1	0	4	13	5	10	6	39
Lalynx(C32)	0	0	1	1	2	2	3	1	10
Skin(C43,C44)	0	0	0	2	1	0	0	2	5
Others	5	14	21	28	59	46	54	34	261
Female									
Total deaths	83	136	236	425	713	944	1,099	1,304	4,940
Cancer deaths	45	66	129	213	331	335	336	275	1,730
%	54.2	48.5	54.7	50.1	46.4	35.5	30.6	21.1	35.0
Stomach(C16)	7	10	14	43	51	58	56	48	287
Lung(C33,C34)	6	3	15	24	44	40	43	30	205
Liver(C22)	1	4	8	22	33	49	21	23	161
Pancreas(C25)	1	5	10	17	35	29	34	25	156
Colon(C18)	0	5	12	17	23	34	26	34	151
Gall bladder/bile duct(C23,C24)	3	9	9	15	20	27	38	27	148
Breast(C50)	9	13	14	16	17	11	10	4	94
Uterus(C53-C55)	6	2	12	9	12	11	13	14	79
Rectum(C19,C20)	2	4	5	4	19	8	13	7	62
Malignant lymphoma(C81-C85)	1	1	4	10	10	7	10	4	47
Ovary(C56)	4	4	9	6	17	5	6	5	56
Esophagus(C15)	0	1	2	1	2	6	5	6	23
Bladder(C67)	0	0	0	1	4	3	7	6	21
Mouth(C00-C14)	0	0	2	3	1	2	6	5	19
Skin(C43,C44)	0	0	1	2	1	0	1	3	8
Lalynx(C32)	0	0	0	0	1	1	0	0	2
Others	5	5	12	23	41	44	47	34	211

in the parentheses: code of ICD 10th

Table 3. Sex-specific satandardized mortality raios (SMRs) and 95% confidence intervals (CI) of total deaths, all cancer deaths, and site-specific cancers by district.

	Hokkaido	Tohoku	Kanto	Chubu	Kinki	Chugoku	Kyushu	Total
	SMR 95%CI	SMR 95%CI	SMR 95%CI	SMR 95%CI	SMR 95%CI	SMR 95%CI	SMR 95%CI	SMR 95%CI
				Male				
Total dealth	61 (52, 72)	84 (79, 89)	77 (71, 83)	75 (72, 78)	80 (75, 84)	79 (75, 84)	84 (79, 90)	78 (76, 80)
All Cancer death	72 (57, 90)	87 (78, 96)	76 (66, 87)	73 (68, 78)	82 (75, 90)	83 (75, 92)	97 (87, 106)	81 (78, 84)
Cancer site:								
Lung (C33,C34)*	95 (59, 145)	102 (83, 124)	83 (62, 109)	73 (63, 85)	115 (97, 136)	78 (62, 97)	84 (66, 105)	87 (81, 94)
Stomach (C16)	62 (33, 106)	82 (65, 103)	100 (76, 130)	86 (75, 99)	80 (64, 98)	64 (48, 82)	96 (77, 119)	83 (77, 90)
Liver (C22)	34 (11, 79)	61 (43, 83)	64 (41, 94)	56 (45, 69)	58 (43, 78)	129 (101, 161)	150 (121, 185)	78 (70, 87)
Pancreas (C25)	120 (48, 247)	115 (77, 165)	82 (44, 141)	63 (45, 86)	97 (66, 138)	86 (54, 131)	78 (47, 122)	84 (71, 98)
Colon (C18)	78 (25, 182)	57 (33, 93)	28 (9, 65)	75 (56, 98)	45 (26, 73)	83 (53, 124)	94 (61, 139)	67 (57, 79)
Gall bladder/bile duct (C23,24)	125 (41, 291)	129 (82, 194)	145 (82, 234)	104 (76, 138)	71 (41, 115)	110 (68, 168)	71 (37, 124)	102 (86, 121)
Rectum (C19,C20)	23 (1, 128)	97 (57, 153)	59 (24, 122)	92 (66, 123)	81 (48, 126)	71 (38, 122)	122 (77, 185)	87 (72, 104)
Esophagus (C15)	101 (33, 235)	118 (77, 175)	30 (8, 77)	59 (40, 83)	41 (21, 74)	69 (38, 116)	88 (52, 139)	68 (56, 82)
Prostate (C61)	95 (20, 277)	103 (58, 169)	65 (24, 142)	97 (68, 134)	58 (29, 105)	109 (65, 170)	89 (46, 154)	90 (73, 109)
Malignant lymphoma (C81-C85)	130 (27, 380)	79 (34, 156)	94 (34, 205)	71 (42, 112)	70 (32, 133)	97 (47, 179)	93 (43, 177)	82 (63, 105)
Mouth (C00-C14)	57 (1, 317)	147 (73, 262)	63 (13, 184)	48 (22, 91)	64 (23, 140)	170 (87, 295)	99 (40, 204)	87 (65, 115)
Bladder (C67)	65 (2, 362)	57 (16, 146)	114 (37, 266)	44 (19, 87)	89 (38, 175)	63 (20, 147)	120 (52, 236)	71 (51, 97)
Lalynx (C32)	0 —	0 —	0 —	77 (25, 179)	62 (8, 224)	37 (1, 206)	81 (10, 292)	51 (24, 94)
Skin (C43, C44)	0 —	196 (24, 708)	0 —	38 (1, 212)	77 (2, 429)	0 —	100 (3, 557)	63 (20, 147)
				Female				
Total dealth	66 (55, 79)	72 (67, 78)	71 (64, 79)	69 (65, 72)	69 (64, 74)	61 (57, 65)	74 (68, 79)	69 (67, 70)
All Cancer death	75 (55, 99)	79 (69, 89)	75 (63, 89)	80 (74, 87)	63 (55, 72)	72 (64, 80)	83 (74, 93)	76 (72, 79)
Cancer site:								
Stomach (C16)	28 (6, 82)	80 (57, 110)	91 (59, 133)	79 (63, 97)	73 (52, 99)	58 (41, 79)	93 (70, 122)	76 (67, 85)
Lung (C33,C34)	52 (14, 133)	63 (40, 96)	99 (61, 151)	65 (49, 85)	60 (39, 89)	87 (64, 117)	89 (62, 124)	74 (64, 85)
Liver (C22)	82 (27, 191)	86 (54, 128)	50 (22, 99)	70 (51, 95)	35 (18, 63)	86 (59, 120)	133 (95, 183)	78 (66, 91)
Pancreas (C25)	134 (49, 292)	89 (53, 140)	106 (56, 181)	135 (104, 172)	75 (44, 118)	67 (41, 103)	74 (43, 119)	97 (82, 113)
Colon (C18)	137 (59, 270)	111 (74, 160)	75 (39, 132)	75 (55, 100)	55 (32, 88)	61 (39, 91)	54 (31, 87)	73 (62, 85)
Gall bladder/bile duct (C23,24)	131 (48, 286)	80 (46, 128)	124 (71, 201)	90 (66, 120)	82 (51, 125)	67 (42, 102)	83 (50, 128)	86 (73, 101)
Breast (C50)	79 (21, 202)	51 (24, 93)	50 (18, 109)	64 (42, 93)	45 (21, 82)	98 (61, 150)	68 (39, 110)	64 (52, 79)
Uterus (C53-C55)	0 —	72 (33, 137)	80 (29, 174)	71 (43, 110)	104 (58, 171)	98 (56, 159)	89 (47, 151)	82 (65, 102)
Rectum (C19,C20)	75 (9, 271)	94 (47, 170)	57 (16, 146)	56 (31, 93)	29 (8, 74)	81 (43, 139)	96 (51, 165)	68 (52, 87)
Ovary (C56)	37 (1, 206)	27 (6, 79)	61 (17, 156)	113 (75, 165)	32 (9, 82)	84 (42, 150)	48 (18, 105)	68 (52, 89)
Malignant lymphoma (C81-C85)	197 (41, 575)	58 (16, 148)	24 (1, 134)	69 (34, 123)	123 (58, 224)	109 (54, 195)	90 (36, 185)	86 (63, 114)
Esophagus (C15)	0 —	80 (16, 234)	88 (11, 318)	78 (31, 161)	156 (63, 321)	54 (11, 158)	23 (1, 128)	76 (48, 115)
Bladder (C67)	0 —	73 (9, 264)	117 (14, 422)	128 (59, 243)	29 (1, 162)	65 (13, 190)	127 (35, 325)	90 (56, 138)
Mouth (C00-C14)	0 —	211 (77, 460)	0 —	107 (43, 220)	89 (18, 260)	50 (6, 181)	31 (1, 173)	85 (51, 133)
Skin (C43, C44)	0 —	0 0 0	0 —	288 (106, 628)	190 (23, 686)	0 —	0 —	113 (49, 223)
Lalynx (C32)	0 —	404 (10,2250)	0 —	0 —	335 (8,1866)	0 —	0 —	99 (12, 357)

* Code in parenthesis is ICD-10.

(1,730) in male and female total deaths (7,238 and 4,940) as shown in Table 2. Among male cancer deaths, cancers of the lung (code of ICD 10th: C33, C34), stomach (C16), and liver (C22) were the three commonest sites, accounting for 23.0%, 20.8%, and 13.1%, respectively. In women, the three commonest sites were cancers of the stomach, lung, and liver, accounting for 16.6%, 11.8%, and 9.3%, respectively. The commonest were the stomach, large intestine (12.3%), and lung when the large intestine was defined by the cancer of colon and rectum (C18-20).

Sex-specific SMRs of total deaths, all cancer deaths, and site-specific cancer deaths were shown in Table 3. Site-specific SMRs for a total cohort were less than 100 except for cancer of the gall bladder/bile duct (C23, C24) in males and pancreas (C25) and skin (C43, C44) in females. SMRs of total deaths and all cancer deaths by district were less than 100 in both males and females. Most of the SMRs by cancer site and district were less than 100 though some exceeded this.

DISUCUSSION

The follow-up condition of cancer deaths by site as of the end of 1999 was almost same as of the end of 1997.[1] The three commonest sites, cancers of the lung, stomach, and liver in males were of the same order and almost the same proportion among all cancer deaths as of the end of 1997.[1] Among females, the three commonest sites, cancers of the stomach, large intestine, and lung were same in each site as of the end of 1997,[1] but the proportion of cancer of the large intestine (12.3%) exceeded that of the lung (11.8%) if the cancer of the colon and rectum were combined.

SMRs of total deaths, all cancer deaths, and most site-specific cancers were less than 100 in both males and females. This means that our cohort members appeared to be less likely to die from total causes and cancers in comparison with the Japanese population as observed other Japanese cohort.[8] Because some of the cohort members were selected from participants in health check-ups or other kinds of screening, they might have had slightly healthier lifestyles that prevented them from dying with lifestyle related diseases such as cancers and cerebrovascular diseases. Cohort members of each district also appeared to be slightly healthier than the general population, as most SMRs of total deaths and site-specific cancer deaths by district were less than 100. It might be due to the small cohort size that some SMRs of site-specific cancer deaths by district were more than 100. Even though our cohort members were slightly healthier than the general Japanese population in the study period, internal comparisons between an exposed group and a group of unexposed to any factors within the cohort can also be justified as a cohort study.

MEMBER LIST OF THE JACC STUDY GROUP

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Stability of Frozen Serum Levels of Insulin-like Growth Factor-I, Insulin-like Growth Factor-II, Insulin-like Growth Factor Binding Protein-3, Transforming Growth Factor β, Soluble Fas, and Superoxide Dismutase Activity for the JACC Study

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BACKGROUND: Subjects of the Japan Collaborate Cohort Study (JACC Study) gave peripheral blood samples collected between 1988 and 1990. We conducted to investigate whether levels of serum components measured after 9 years of frozen storage are stable or not.
METHODS: To assess the degradation of frozen serum components in the JACC Study, we compared levels of various components (IGF-I, IGF-II, IGFBP-3, TGF-β1, sFas, and total SOD activity) between fresh and stored sera collected from other inhabitants. Serum levels of constituents were measured by immunoradiometric assay (IGF-I, IGF-II and IGFBP-3), quantitative enzyme immunoassay (TGF-β1), enzyme-linked immuno-adsorbent assay (sFas), and an improved nitrite method (SOD activity).
RESULTS: The coefficients of variation for intra- and inter-assay precisions of the measurements were less than 9%. Levels of IGF-I, IGF-II, IGFBP-3, TGF-β1 and sFas in sera after storage for 9 years at -80℃ were similar to those of fresh sera newly collected from inhabitants. The distributions of serum IGF-I, IGF-II, IGFBP-3, TGF-β1, sFas and SOD activity for specimens collected from different individuals tended to be similar to those of serum levels for frozen specimens collected from different individuals and stored for 9 years.
CONCLUSIONS: There was no statistically significant difference in distribution of measured values of IGF-I, IGF-II, IGFBP-3, TGF-β1, and sFas between newly collected sera and frozen specimens stored for 9 years. Thus, measurements of these serum constituents of specimens stored for the JACC Study can be reliably used in nested case-control study.
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Key words: Serum Storage, IGFs, sFas, TGF-β, SOD

The Japan Collaborate Cohort Study for Evaluation of Cancer Risk (JACC Study), sponsored by Monbusho (the Ministry of Education, Science, Sports and Culture of Japan), involves more than 127,477 participants living in 45 municipalities all over Japan.[1,2] Subjects of the JACC Study completed a survey and gave peripheral blood samples collected from 39,242 registered subjects (aged from 40 to 79 years) between 1988 and 1990. Serum from these samples was separated from blood cells and stored in deep freezers at -80℃ until 1999; serum of each participant was divided into 3 to 5 tubes (100 to 500 μL per tube). These serum

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samples are used to study the relation between serum component levels and the incidence or mortality of cancer or other diseases.

Recently, cancer prevention research has focused on molecular biology related to genes, cytokines and special molecules associated with the promotion or inhibition of the development of carcinogenesis and apoptosis. There were some reports of the relationship investigated between cancer and the following serum constituents: insulin-like growth factor (IGF)-I, IGF-II,[3-5] insulin-like growth factor-binding protein 3 (IGFBP-3),[6] transforming growth factor (TGF)-β 1,[7,8] soluble Fas (sFas),[9] and superoxide dismutase (SOD) activity.[10-13] Although reports indicate that serum levels of constituents such as proteins and minerals are stable in long-term refrigerated storage,[14-17] there have been no reports indicating whether serum levels of cytokines, such as IGFs, TGF-β and sFas, remain stable after approximately 10 years of storage at -80℃.

In the present study, we examined whether cytokines and other constituents in frozen sera remained stable during long-term storage at -80℃, using stored and fresh serum samples separately collected from other subjects.

METHODS

Serum Samples

Approximately 2 liters of pooled sera prepared to evaluate the stability of serum biochemical constituents for the JACC Study was collected from individuals who participated in health check-up programs for workers in certain industries. After centrifugation and filtration 3 times, 1mL samples of the pooled sera were put into 2-ml cups that were sealed with a polypropylene stopper, distributed to laboratories and stored at -80℃ beginning in 1988. Fresh sera used for comparison of serum levels between fresh and frozen sera were separately collected from inhabitants of rural Saitama (1999) and Hokkaido (1990) who participated in health check-up programs. Serum samples used for comparison of serum level of inhabitants, aged 40 to 80, were collected from residents of Hokkaido who attended health check-up programs in August 1991 and 1999. Approximately 3mL serum samples were poured into polypropylene cups sealed with a polypropylene stopper, and were stored at -80℃ until the time of measurement of components. Reference sera for intra- and inter-assay precisions were used different levels of controlled specimens or pooled sera specially prepared by SRL Laboratory (SRL Laboratory, Hachioji).

Measurements of serum constituents

We measured serum levels of IGF-I, IGF-II, IGFBP-3, TGF-β 1, sFas and total SOD activity. All measurements were performed using the same batched-reagent set, by trained staff at a single laboratory (SRL Laboratory, Hachioji). Measurements used for comparison between fresh and stored sera were performed in 1999. Serum component levels of pooled sera were measured using a SMAC auto-analyzer (Technicon Co., Ltd.; for measurements in 1988) and a TBA auto-analyzer (Toshiba K.K.; for measurements in 1994).

Serum levels of IGF-I, IGF-II and IGFBP-3 were measured by immuno-radiometric assay, using commercially available kits (Daiichi Radioisotope Lab., Tokyo)[18-20] (Table 1). Serum TGF-β 1 was measured by quantitative sandwich enzyme immunoassay (ELISA), using commercially available kits (R&D Systems Inc., Minneapolis).[21,22] Serum sFas was assayed by enzyme-linked immuno-adsorbent assay (ELISA), using commercially available kits (MBL Co., Ltd., Nagoya).[23,24] Serum SOD activity was estimated from the decreasing rate of nitrite produced by hydroxylamine and superoxide anions, based on an improved nitrite method.[25]

The coefficients of variation (CV) of intra- and inter-assay precisions for each determination were calculated: from 10 determinations of 3 different reference sera for intra-assay precision; and from 5-day determinations of 5 different reference sera for inter-assay precision. Each range of CV values estimated with different reference sera was presented as the lowest and highest mean values. The ranges of inter-assay precision for the JACC Study were represented as the low and high CV values calculated from the reference serum levels estimated in each assay of the JACC Study samples. Paired t-tests were performed to evaluate the mean differences between fresh and frozen sample levels.

Our entire study design, which comprised singular and collective use of epidemiological data and sera, was approved by the Ethical Board at Nagoya University School of Medicine, where the central secretariat of the JACC study is located.

RESULTS

The range of the assays for reliable measurement of IGF-I, IGF-II and IGFBP-3 in reference sera was 4 to 2,000 ng/mL, 10 to 1,640 ng/mL, and 0.06 to 10.10 μ g/mL, respectively (Table 1). The intra- and inter-assay precisions obtained using different reference sera for each determination method was as follows: for the IGF-I assay, 2.15 to 3.53% and 1.12 to 4.18% of the CV values, respectively; for the IGF-II assay, 2.74 to 4.45% and 4.23 to 5.53%; for the IGFBP-3 assay, 3.16 to 4.19% and 5.28 to 8.89%. The range of the assay for serum TGF-β 1 level was 16 to 2,178 ng/mL; the intra- and inter-assay precisions were 2.67 to 6.79% and 4.17 to 6.16% of the CV values, respectively. The range of the assay for serum sFas level was 5.0 to 50 pg/ml; the intra- and inter-assay precisions were 2.18 to 5.55% and 8.24 to 12.30%, respectively. The range of the assay for serum SOD activity was 0.1 to 10.0 U/ml; the intra- and inter-assay precisions were 4.02 to 6.79% and 2.79 to 5.82%, respectively. Mean day-to-day variations (inter-assay precision) of reference sera estimated at the time of measurements for the JACC Study samples were 2.30% for IGF-I, 8.74% for IGFBP-3, 7.51% for TGF-β 1, 7.91% for sFas, , and 8.77% for SOD activity.

Table 2 shows the comparison of serum component levels between fresh samples and samples stored for 6 years at -80℃. There were no apparent differences in serum levels of proteins or

Table 1. Determination method and its precision when serum levels of IGFs, IGF-BP3, TGF- β 1, sFas, and SOD activity in serum samples were estimated by the method used in this study.

Item		IGF-I	IGF-II	IGFBP-3	TGF- β 1	sFas	SOD activity
Assay method		Immuno-radiometric assay (IRMA)	Immuno-radiometric assay (IRMA)	Immuno-radiometric assay (IRMA)	Quantitative sandwich enzyme immunoassay	Enzyme-linked immuno-adsorbent assay (ELISA)	Improved nitrite method (Colorimetric method)
Assay reagents	Company supplied the reagent kit	Daiichi Radioisotope Lab.	Daiichi Radioisotope Lab.	Daiichi Radioisotope Lab.	R&D Systems Inc.	BML Company Ltd.	SRL Lab.
Detection	Ranges (unit)	4-2,000 (ng/mL)	10-1,640 (ng/mL)	0.06-10.10 (μ g/mL)	16-2,178 (ng/mL)	1.0 - 10.0 (pg/mL)	0.1 - 10.0 (U/mL)
Precision	Intra-assay(%)	2.15 - 3.53	2.74 - 4.45	3.16 - 4.19	2.67 - 6.79	2.18 - 5.55	4.02 - 6.79
	Inter-assay(%)	1.12 - 4.18	4.23 - 5.53	5.28 - 8.89	4.17 - 6.16	8.24 - 12.30	2.79 - 5.82
Assay precision	Inter-precision for JACC Study	2.30	-	8.74	7.51	7.90	8.77

Reference serum (CV%): Coefficients of variation were caliculated from the mean values of reference sera estimated by each assay for JACC Study samples.

Table 2. Comparison of serum costituent values in pooled serum determined between 1988 and 1994.

	Year of determination	
Serum component	1988	1994
Total protein (g/dL)	7.5	7.1
Albumin (g/dL)	3.8	4.0
Total bilirubin (mg/dL)	0.7	0.5
Urea (mg/dL)	22	21
Uric acid (mg/dL)	4.9	5.7
Creatinine (mg/dL)	1.5	1.3
Total cholesterol (mg/dL)	209	189
Triglyceride (mg/dL)	121	109
Sodium (mEq/L)	146	146
Potassium (mEq/L)	4.4	4.4
Chloride (mEq/L)	108	102
Inorganic phosphate (mg/dL)	3.9	3.9
Calcium (mg/dL)	8.5	8.0
GOT (IU/L)	16	19
GPT (IU/L)	14	14
LDH (IU/L)	64	75
ALP (IU/L)	52	39
CHE (IU/L)	4,074	3,520
γ -GTP (IU/L)	46	42
LAP (IU/L)	94	77
Amylase (IU/L)	28	23
Autoanalyzer	SMAC (Technicon Co., Ltd.)	TBA (Toshiba K.K.)

1988: data estimated from fresh pooled serum at the time of preparation.
1994: data estimated from pooled serum stored during a 6-year storage at -80℃.

Table 3. Comparison of serum levels of certain cytokines and SOD activity between fresh and frozen samples.

Sample	IGF-I (ng/mL)	IGF-II (ng/mL)	IGFBP-3 (μ g/mL)	TGF- β 1 (ng/mL)	sFas (ng/mL)
Fresh sample	199.8 (21.5)	665.7 (50.9)	3.01 (0.11)	32.99 (3.36)	1.92 (0.23)
N	10	10	10	10	10
Frozen sample	186.2 (12.7)	616.3 (29.7)	3.12 (0.25)	30.44 (2.43)	1.77 (0.22)
N	21	21	21	20	19
Probability	p =0.64	p =0.48	p =0.64	p =0.55	p =0.81

Fresh sample: determination at the time of serum collection in 1999 .

Frozen sample: determination of serum samples collected in 1990 after 9-year storage at -80℃.

Table 4. Comparison of serum levels of IGFs, IGFBP-3, TGF- β 1, sFas, and SOD activity in 100 inhabitants (46 males and 64 females, aged 39-78) collected between 1991 and 1999.

Component		Collected year		Mean
		1991	1999	differences
IGF-I	Mean	167	162	-5
(ng/mL)	25%	130	120	(-3.0%)
	50%	160	150	
	75%	200	200	
IGF-II	Mean	649	652	3
(ng/mL)	25%	570	560	(-0.5%)
	50%	630	660	
	75%	728	750	
IGFBP-3	Mean	3.09	3.03	-0.06
(μ g/mL)	25%	2.72	2.55	(-1.9%)
	50%	3.09	3.07	
	75%	3.52	3.51	
IGF- β 1	Mean	32.3	36.9	4.6
(ng/mL)	25%	22.5	31.6	(14.2%)
	50%	32.0	36.7	
	75%	43.6	42.2	
sFas	Mean	2.44	2.64	0.20
(ng/mL)	25%	1.40	1.6	(8.2%)
	50%	1.70	1.85	
	75%	2.00	2.2	
SOD Activity	Mean	2.9	2.5	-0.4
(U/mL)	25%	1.63	1.8	(-13.8%)
	50%	1.9	2.1	
	75%	2.2	2.5	

Data represented as mean (mean value) and ranges (25%, 50% and 75%).

Mean difference: difference value = 1999-level - 1991-level.

Difference precentages (%) = difference value/ 1991-level.

minerals, although there were decreases in serum levels of organic compounds including bilirubin, lipids and some enzyme activities, but serum levels of uric acid, GOT and LDH activities tended to increase, because of bias due to use of different auto-analyzers.

Serum values of IGF-I, IGF-II, IGFBP-3, TGF-β, and sFas in individual sera after storage for 9 years at -80℃, which were separately collected from Hokkaido inhabitants in 1990, were similar to those of fresh sera newly collected from Saitama healthy inhabitants in 1999 (Table 3). In a study of other serum samples collected from 100 healthy individuals (46 males and 64 females) in 1991 and 1999, and stored at -80℃ until 2000, there tended to be similarity in distribution of serum values of IGF-I, IGF-II, IGFBP-3 and sFas between serum samples collected in 1991 and 1999 from different individuals, although those of TGF β 1 and SOD activity tended to change during storage (Table 4).

DISCUSSION

In the JACC Study, sera collected from 39,242 subjects and separated from blood cells were stocked in deep freezers at -80℃ until 1999; serum of each participant was divided into 3 to 5 tubes (100 to 500 μL per tube). We were unable to assess the stability of serum samples stored for about 10 years, because the volume of serum samples for the JACC Study was insufficient for measurements of many constituents using different various protocols. Therefore, we evaluated the stability of frozen and stored sera using serum samples separately collected from other inhabitants and pooled serum.

Results of this study, in which values were compared between fresh and frozen samples of pooled serum prepared for quality control of determination of JACC Study samples, demonstrate that serum levels of proteins such as albumin and total protein tend to remain steady during frozen storage for several years.

Some reports indicate that there is little change in serum levels of constituents such as proteins, minerals, glucose and uric acid during storage at -70℃,[14] although serum levels of creatinine and lipids such as triglyceride tended to decrease during storage for 6 years at -80℃ in this study. The difference in enzyme activities in the present study may be due to the estimation methods used for each auto-analyzer, although it has been reported that serum AST (GOT) activity changes during storage.[14,15] It has also been reported that the plasma protein fraction can be safely used after storage for 5 years at room temperature.[17] There have been no previous detailed reports about the stability of cytokines such as IGFs, TGF-β 1 and sFas in frozen serum samples examined after long-term storage. In the present study, the mean values of cytokines such as TGF-β 1 in sera separately collected from inhabitants varied over a range of about 14% after 9 years of storage at -80℃. However, we also obtained that serum values of other cytokines such as IL-6 (but not TNF-α) tended to decrease (more than 60%) after 9 years of storage at -80℃, in comparison between fresh and frozen samples. The range of variation was similar to the reported coefficients of variation for determinations of IGFs, TGF-β, sFas and SOD activity: 1.1 to 7.3% for intra-assay, and 1.6 to 11.7% for inter-assay.[18-25] Moreover, in the present study, serum SOD activity was stable during long-term storage at -80℃. In previous studies of SOD activity, there was no significant change related to storage time or temperature,[12,26] erythrocyte-SOD activity was unstable,[27] and protein levels were unusually stable.[28]

The present results indicate that serum levels of IGF-I, IGF-II, IGFBP-3, sFas, and TGF-β 1 remain stable during long-term storage at -80℃, because distributions of serum levels of these constituents were nearly equal between fresh and frozen specimens separately collected from different inhabitants. They also suggest that SOD activity is a useful biomarker for cancer prevention research such as a nested case-control study.

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Serum Levels of Polyunsaturated Fatty Acids and Risk of Colorectal Cancer: A Prospective Study

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To examine the relation between serum fatty acids and risk of colorectal cancer, the authors conducted a nested case-control study of 169 colorectal cancer cases and 481 controls matched by age and enrollment area as part of the Japan Collaborative Cohort Study. Serum samples were donated by subjects at baseline (between 1988 and 1990) and were stored at −80°C until 2002. Serum fatty acid levels were measured by using gas chromatography and were expressed as the weight percentage of total lipids. Conditional logistic regression analyses adjusted for lifestyle factors revealed that total ω-3 polyunsaturated fatty acids (odds ratio = 0.24, 95% confidence interval: 0.08, 0.76), α-linolenic acid (odds ratio = 0.39, 95% confidence interval: 0.16, 0.91), docosapentaenoic acid (odds ratio = 0.30, 95% confidence interval: 0.11, 0.80), and docosahexaenoic acid (odds ratio = 0.23, 95% confidence interval: 0.07, 0.76) all showed a significantly decreased risk for the highest versus the lowest quartile levels for colorectal cancer in men. For women, a weak negative association was observed between docosapentaenoic acid and colorectal cancer risk, although it was not statistically significant. No adverse effects of high serum levels of ω-6 polyunsaturated fatty acids on colorectal cancer risk were detected.

alpha-linolenic acid; chromatography; colorectal neoplasms; docosahexaenoic acids; eicosapentaenoic acid; fatty acids; prospective studies; serum

Abbreviations: CI, confidence interval; JACC Study, Japan Collaborative Cohort Study for the Evaluation of Cancer Risk; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acid; Q, quartile.

A number of experimental studies have reported an association between specific fatty acids and colorectal cancer risk. In particular, protective effects of ω-3 polyunsaturated fatty acids (PUFAs) (1–5) and adverse effects of ω-6 PUFAs (6–8) have been observed. However, the evidence from epidemiologic studies is limited and inconsistent (9). One major problem is the difficulty of measuring fatty acids accurately. The fatty acid composition of serum lipids is considered a reliable index reflecting dietary intake of fatty acids over periods of weeks or months (10, 11). Nevertheless, because of the high cost of measuring serum fatty acid levels, this procedure is performed in only those studies with relatively small numbers of subjects. Here, we report the results of a nested case-control study conducted as part of a nationwide

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cohort study, in which the association between serum fatty acid levels and risk of colorectal cancer was examined prospectively.

MATERIALS AND METHODS

Subjects

Details of the Japan Collaborative Cohort Study for the Evaluation of Cancer Risk (JACC Study), sponsored by Monbukagakusho (the Ministry of Education, Culture, Sports, Science and Technology of Japan), have been reported elsewhere (12, 13). The JACC Study involved 110,792 healthy residents who were aged 40–79 years at baseline and were recruited from 45 areas throughout Japan between 1988 and 1990. The subjects for the present study were restricted to 65,184 persons who lived in 24 study areas in which cancer registries were available. On enrollment, the participants completed a self-administered questionnaire that assessed demographic characteristics, lifestyle, and medical history. Blood samples were donated by 36.6 percent of the total study group (n = 23,863). Participants who reported a previous history of cancer (n = 409) were excluded from the analysis. Written informed consent for participation was obtained individually from most subjects, with the exception of those in study areas in which informed consent was provided at the group level after the aim of the study and the confidentiality of the data had been explained to community leaders. The study protocol was approved by the Ethics Committee of Medical Care and Research of the Fujita Health University School of Medicine, Japan.

Case ascertainment and control selection

Cases were defined as subjects who developed colorectal cancer (according to the *International Statistical Classification of Diseases and Related Health Problems*, Tenth Revision, codes C18–20) during the follow-up period (mean, 7.1 years; standard deviation, 2.3), which ran until the end of 1997. We ascertained the incidence of cancer from population-based cancer registries, supplemented by a systematic review of death certificates (14). For each case, two or three controls with no previous history of cancer were selected from the population at risk. The controls were matched to each case by sex, age (±3 years, as close as possible), and participating institution. A total of 169 colorectal cancer cases (83 men and 86 women) and 481 controls (241 men and 240 women) were involved in the analysis. The numbers of colon cancer cases and matched controls were 119 (52 men and 67 women) and 336 (151 men and 185 women), respectively.

Serum fatty acid analysis

Serum was separated from the blood samples at local laboratories in or near the surveyed municipalities and was stored for 11–14 years at −80°C. All samples were analyzed in November 2002 in one laboratory by trained staff blinded to case-control status. Samples were organized in batches of up to 50, which included two samples from a standard pool for quality control. Lipids in 0.2 ml of serum were extracted with Folch's solution under a nitrogen atmosphere (15). After methyl esterification by 0.4 M potassium methoxide and 14 weight percentage boron trifluoride methanol, fatty acids were measured by using a gas chromatograph (model GC17A; Shimazu, Kyoto, Japan) equipped with an Omegawax 250 capillary column (30-m × 0.25-mm inside diameter; 0.25-μm thickness; Supelco, Bellefonte, Pennsylvania). Peaks were determined by using a flame-ionization detector and were quantified with an electric integrator (model CR-7A; Shimazu) using pure standard mixtures (Sigma, St. Louis, Missouri).

A total of 24 fatty acids were identified from 12:0 to 24:1 ω-9. The serum level of each fatty acid was expressed as the composition, by weight percentage, of total lipids. The limit of detection for the assay was 0.02 weight percent. The respective repeatability and day-to-day variation of the standard sample coefficients of variation were as follows: 5.5 percent for both measurements for 16:0; 6.2 percent and 5.6 percent for 18:2; 4.9 percent and 6.5 percent for 20:3; and 2.9 percent and 4.5 percent for 24:1.

In particular, four ω-3 PUFAs and six ω-6 PUFAs were measured: α-18:3 ω-3 (α-linolenic acid), 20:5 ω-3 (eicosapentaenoic acid), 22:5 ω-3 (docosapentaenoic acid), 22:6 ω-3 (docosahexaenoic acid), 18:2 ω-6 (linoleic acid), γ-18:2 ω-6 (γ-linolenic acid), 20:2 ω-6 (eicosadienoic acid), 20:3 ω-6 (dihomo-γ-linolenic acid), 20:4 ω-6 (arachidonic acid), and 22:4 ω-6 (docosatetoraenoic acid). In addition, we calculated the content of total saturated fatty acids (12:0 + 14:0 + 16:0 + 18:0 + 20:0 + 22:0 + 24:0), monounsaturated fatty acids (MUFAs; 16:1 ω-7 + 18:1 ω-9 + 20:1 ω-9 + 22:1 ω-9 + 24:1 ω-9), and total ω-3 and ω-6 PUFAs. The ratio of ω-6 to ω-3 PUFAs was also determined.

Statistical methods

Background characteristics were compared between cases and controls by using the Cochran-Mantel-Haenszel test (16) and analysis of covariance (17), with adjustment for matching factors (age and area of enrollment) by sex. Spearman's correlation coefficients were calculated to determine the association between fatty acids. Conditional logistic regression models were used to calculate odds ratios for the incidence of colorectal cancer (18) for the serum level of each specific fatty acid. The odds ratios for colon cancer risk were also examined separately from those for rectal cancer. Cases and controls were divided into four groups according to the level of fatty acids in controls. Odds ratios were calculated for the second quartile ((Q)2), third quartile (Q3), and highest quartile (Q4) versus the lowest quartile (Q1). To test for linear trends in odds ratios over quartiles, we coded each quartile as 0, 1, 2, or 3 and incorporated these data into the logistic model as a single variable.

We adjusted for the following factors by including them in the logistic models (19): age at completing final education (≤18 years or ≥19 years); history of colorectal cancer in parents or siblings (yes or no); body mass index (weight (kg)/height (m)^2; <20.0 kg/m^2, 20.0–24.9 kg/m^2, or ≥25.0 kg/m^2) calculated from reported height and weight at baseline; smoking status (never, former, or current); daily alcohol

TABLE 1. Baseline characteristics of colorectal cancer cases and controls from the Japan Collaborative Cohort Study for the Evaluation of Cancer Risk, 1988–1997

Variable	Men		Women	
	Cases (n = 83)	Controls (n = 241)	Cases (n = 86)	Controls (n = 240)
Age in years (mean (standard deviation))	61.0 (8.0)	60.5 (7.7)	62.7 (7.8)	62.4 (7.8)
Body mass index† (mean (standard deviation))	23.1 (2.7)	22.8 (2.7)	22.9 (3.5)	23.3 (3.2)
History of colorectal cancer in parents or siblings: yes (%)	4.8	1.7	9.3	2.9*
Smoking history (%)				
Current smoker	44.6	46.1	3.5	2.9
Former smoker	34.9	27.8	2.3	2.1
Never smoker	16.9	21.6	87.2	86.7
Unreported	3.6	4.6	7.0	8.3
Alcohol drinking history (%)				
Current drinker	79.5	75.5	23.3	18.8
Former drinker	2.4	3.7	0.0	1.7
Never drinker	16.9	17.5	73.3	74.2
Unreported	1.2	3.3	3.4	5.3
Intake frequency of green leafy vegetables (e.g., spinach): almost every day (%)	56.6	58.5	53.5	56.3
Physical exercise ≥3 hours per week (%)	15.7	16.2	8.1	12.1
Education: age ≥19 years at completion of full-time education (%)	15.7	12.9	9.3	8.3

* $p < 0.05$ by the Cochrane-Mantel-Haenzel test adjusted for age and area of enrollment.
† Weight (kg)/height $(m)^2$.

consumption (never, former, or current); frequency of intake of green leafy vegetables (almost every day or ≤3–4 days per week), and time spent exercising (<3 hours per week or ≥3 hours per week) (20). The results were not significantly affected by adjustment for potential confounding factors; therefore, only the multivariate-adjusted odds ratios are presented in the tables in this paper. To eliminate the influence of undiagnosed colorectal cancers at baseline, the analyses were repeated by excluding men and women who developed colorectal cancer within the first 2 or 5 years of follow-up, respectively, along with their matched controls.

All analyses were performed by using SAS software, release 8.2 (SAS Institute, Inc., Cary, North Carolina). In the conditional logistic regression analysis, missing values for each categorical covariate were treated as an additional category of the variable and were included in the model. Two-tailed probability (p) values of <0.1 were considered marginally significant, and p values of <0.05 were considered statistically significant.

RESULTS

Table 1 gives the baseline characteristics of all colorectal cancer cases and controls by sex. For both men and women, the age distributions of the cases and controls were well matched. Cases were more likely than controls to have a family history of colorectal cancer. There were no significant differences between cases and controls regarding body mass index, educational level, smoking and alcohol drinking habits, or frequency of green leafy vegetable intake or physical exercise.

Spearman's correlation coefficients between the fatty acids were computed (data not shown). The directions of the associations were not affected by sex, and all correlation coefficients were statistically significant. For both men and women, ω-3 PUFAs were mildly inversely correlated with ω-6 PUFAs (r = –0.24 and r = –0.12, respectively), MUFAs (r = –0.30 and r = –0.44, respectively), and saturated fatty acids (r = –0.18 and r = –0.27, respectively). In men and women, ω-6 PUFAs were moderately inversely correlated with MUFAs (r = –0.68 for both sexes) and saturated fatty acids (r = –0.72 and r = –0.77, respectively). In addition, MUFAs and saturated fatty acids were mildly positively correlated in both men and women (r = 0.35 and r = 0.46, respectively).

Table 2 shows the associations between the serum levels of each group of fatty acids and the risk of colorectal cancer by sex. For men, total saturated fatty acids and ω-6 PUFAs failed to show significant associations with colorectal cancer risk. A marginally significant positive trend was observed between serum level of total MUFAs and colorectal cancer risk (p for linear trend = 0.06). Total ω-3 PUFAs were inversely associated with colorectal cancer risk, showing a 76 percent risk reduction when Q4 and Q1 were compared (odds ratio = 0.24, 95 percent confidence interval (CI): 0.08, 0.76; p for linear trend = 0.08). For the ω-6/ω-3 ratio, Q2 showed a marginally significant association, with a 2.36-fold increased risk of colorectal cancer relative to Q1 (95 percent CI: 0.99, 5.66), although the dose-response relation was

TABLE 2. Associations of serum level of fatty acids with colorectal cancer risk, by sex, Japan Collaborative Cohort Study for the Evaluation of Cancer Risk, 1988–1997

Q*	Men						Women					
	Value†	Cases (no.)	Controls (no.)	OR*,‡	95% CI*	*p* trend	Value†	Cases (no.)	Controls (no.)	OR‡	95% CI	*p* trend
						Saturated fatty acids						
Q1	<31.91	18	60	1.00		0.36	<31.40	24	60	1.00		0.17
Q2	31.91–33.73	20	60	1.22	0.51, 2.91		31.40–33.00	27	60	1.10	0.53, 2.32	
Q3	33.74–36.04	20	59	1.12	0.47, 2.64		33.01–35.40	16	60	0.56	0.24, 1.30	
Q4	≥36.05	25	62	1.71	0.66, 4.47		≥35.41	19	60	0.59	0.23, 1.52	
						Monounsaturated fatty acids						
Q1	<20.78	13	50	1.00		0.06	<20.44	24	56	1.00		0.51
Q2	20.78–22.49	13	51	1.04	0.40, 2.74		20.44–22.14	23	57	0.96	0.45, 2.02	
Q3	22.50–24.67	17	50	1.48	0.59, 3.72		22.15–24.12	16	56	0.70	0.30, 1.65	
Q4	≥24.68	28	52	2.05	0.86, 4.89		≥24.13	19	57	0.83	0.36, 1.92	
						ω-3 polyunsaturated fatty acids						
Q1	<7.74	24	60	1.00		0.08	<7.84	24	60	1.00		0.96
Q2	7.74–9.639	19	59	0.76	0.34, 1.72		7.84–9.379	18	60	0.53	0.23, 1.20	
Q3	9.64–12.03	31	61	1.09	0.49, 2.44		9.38–10.96	21	60	0.75	0.35, 1.63	
Q4	≥12.04	9	61	0.24	0.08, 0.76		≥10.97	23	60	0.85	0.38, 1.91	
						ω-6 polyunsaturated fatty acids						
Q1	<28.89	26	60	1.00		0.36	<31.90	23	60	1.00		0.32
Q2	28.89–32.77	19	60	0.79	0.38, 1.64		31.90–34.30	10	59	0.44	0.17, 1.11	
Q3	32.78–36.10	18	60	0.67	0.30, 1.47		34.31–37.53	27	61	1.28	0.58, 2.82	
Q4	≥36.11	20	61	0.69	0.30, 1.61		≥37.54	26	60	1.15	0.48, 2.75	
						ω-6/ω-3 ratio						
Q1	<2.59	14	60	1.00		0.33	<3.07	22	60	1.00		0.90
Q2	2.59–3.369	27	60	2.36	0.99, 5.66		3.07–3.749	20	60	0.84	0.37, 1.92	
Q3	3.37–4.389	19	60	1.76	0.67, 4.64		3.75–4.579	19	60	0.76	0.35, 1.65	
Q4	≥4.39	23	61	2.05	0.78, 5.40		≥4.58	25	60	1.08	0.50, 2.36	

* Q, quartile; OR, odds ratio; CI, confidence interval.

† Values are expressed as the weight percentage of total serum lipids.

‡ Odds ratios were derived from a conditional logistic analysis model adjusted for family history of colorectal cancer in first-degree relatives, body mass index, education, smoking and alcohol drinking history, green leafy vegetable intake, and physical exercise. For men, 83 cases and 241 controls and, for women, 86 cases and 240 controls matched on age and participating institution were involved in the analyses.

unclear. In the analysis of colon cancer risk alone (data not shown), there was no statistically significant association with the serum levels of any of the fatty acids. However, the directions of the nonsignificant trends were similar to those observed in the combined analyses. The odds ratios for Q4 versus Q1 of each group of fatty acids for the risk of colon cancer in men were as follows: 1.00 for saturated fatty acids (95 percent CI: 0.28, 3.52; *p* for linear trend = 0.84), 1.19 for MUFAs (95 percent CI: 0.34, 4.22; *p* for linear trend = 0.60), 0.40 for ω-3 PUFAs (95 percent CI: 0.10, 1.55; *p* for linear trend = 0.43), and 1.04 for ω-6 PUFAs (95 percent CI: 0.34, 3.16; *p* for linear trend = 0.86).

For women, the association between the levels of all groups of fatty acids, except saturated fatty acids, and colorectal cancer risk tended to be U- or J-shaped, although any associations failed to reach the statistically significant level (table 2). Similar results were obtained when the analyses were repeated for colon cancer cases alone (data not shown). The odds ratios for Q4 versus Q1 of groups of fatty acids were as follows: 0.56 for saturated fatty acids (95 percent CI: 0.20, 1.59; *p* for linear trend = 0.12), 0.70 for MUFAs (95 percent CI: 0.26, 1.84; *p* for linear trend = 0.53), 0.90 for ω-3 PUFAs (95 percent CI: 0.37, 2.20; *p* for linear trend = 0.46), and 1.01 for ω-6 PUFAs (95 percent CI: 0.36, 2.86; *p* for linear trend = 0.46).

To exclude the influence of undiagnosed colorectal cancer at baseline, the analyses were repeated by excluding men and women who developed colorectal cancer during the first 2 or 5 years of follow-up, respectively, along with their matched controls (table 3). For men, excluding those who developed colorectal cancer within the first 5 years strengthened both the positive association of total MUFAs and the inverse association of total ω-3 PUFAs with colorectal cancer risk. For women, excluding those who developed colorectal cancer within the first 2 years revealed a significant association for ω-3 PUFAs, with a 70 percent decreased risk of colorectal cancer at the Q2 compared with the Q1 level (odds ratio = 0.30, 95 percent CI: 0.11, 0.79).

Table 4 shows the association of levels of specific ω-3 fatty acids with colorectal cancer risk. For men, of the four

TABLE 3. Associations of serum level of fatty acids with colorectal cancer risk, by length of cases' follow-up period, Japan Collaborative Cohort Study for the Evaluation of Cancer Risk, 1988–1997

Q*,†	Follow-up period for men						Follow-up period for women					
	>2 years (*n* = 64 cases; *n* = 186 controls)			>5 years (*n* = 40 cases; *n* = 115 controls)			>2 years (*n* = 69 cases; *n* = 195 controls)			>5 years (*n* = 30 cases; *n* = 84 controls)		
	OR*,‡	95% CI*	*p* trend	OR‡	95% CI	*p* trend	OR‡	95% CI	*p* trend	OR‡	95% CI	*p* trend
						Saturated fatty acids						
Q1	1.00		0.84	1.00		0.45	1.00		0.31	1.00		0.58
Q2	1.74	0.6, 4.44		1.23	0.32, 4.68		0.59	0.25, 1.39		0.89	0.10, 7.90	
Q3	1.02	0.3, 2.75		1.09	0.23, 5.10		0.43	0.17, 1.09		1.52	0.21, 11.0	
Q4	1.04	0.3, 3.15		1.72	0.33, 9.07		0.69	0.23, 2.02		4.02	0.23, 71.7	
						Monounsaturated fatty acids						
Q1	1.00		0.18	1.00		0.03	1.00		0.72	1.00		0.71
Q2	1.36	0.4, 4.25		2.92	0.51, 16.6		0.99	0.44, 2.20		2.05	0.36, 11.6	
Q3	1.80	0.5, 5.56		1.88	0.33, 10.7		0.67	0.26, 1.73		1.35	0.19, 9.64	
Q4	1.90	0.6, 5.23		8.85	1.37, 57.4		1.00	0.38, 2.60		0.70	0.07, 6.87	
						ω-3 polyunsaturated fatty acids						
Q1	1.00		0.35	1.00		0.09	1.00		0.33	1.00		0.80
Q2	0.82	0.32, 2.08		0.38	0.10, 1.48		0.30	0.11, 0.79		0.04	0.00, 0.63	
Q3	1.46	0.58, 3.68		0.79	0.21, 3.00		0.68	0.29, 1.61		0.01	0.00, 0.51	
Q4	0.30	0.08, 1.16		0.26	0.05, 1.32		0.47	0.18, 1.20		0.59	0.08, 4.52	
						ω-6 polyunsaturated fatty acids						
Q1	1.00		0.68	1.00		0.24	1.00		0.18	1.00		0.66
Q2	1.07	0.45, 2.55		2.03	0.56, 7.33		0.35	0.12, 1.05		0.25	0.01, 5.28	
Q3	0.89	0.36, 2.22		0.75	0.18, 3.12		1.28	0.51, 3.20		3.82	0.33, 43.8	
Q4	0.83	0.32, 2.19		0.54	0.10, 2.78		1.36	0.52, 3.54		1.01	0.13, 8.12	
						ω-6/ω-3 ratio						
Q1	1.00		0.38	1.00		0.36	1.00		0.38	1.00		0.66
Q2	2.57	0.91, 7.29		2.50	0.59, 10.5		1.07	0.41, 2.81		0.95	0.14, 6.62	
Q3	2.07	0.69, 6.23		1.10	0.23, 5.19		0.69	0.27, 1.75		0.60	0.10, 3.79	
Q4	2.12	0.69, 6.54		2.64	0.57, 12.2		1.73	0.71, 4.23		1.74	0.30, 10.2	

* Q, quartile; OR, odds ratio; CI, confidence interval.

† Quartiles were determined by the distribution of each fatty acid, expressed as the weight percentage of total serum lipids, in controls.

‡ Odds ratios were derived from a conditional logistic analysis model adjusted for family history of colorectal cancer in first-degree relatives, body mass index, education, smoking and alcohol drinking history, green leafy vegetable intake, and physical exercise. Cases and controls were matched on age and participating institution.

ω-3 PUFAs examined, all except eicosapentaenoic acid showed significant or marginally significant inverse associations with colorectal cancer risk. Eicosapentaenoic acid narrowly failed to show a significant inverse association with colorectal cancer risk (Q4 vs. Q1 odds ratio = 0.44, 95 percent CI: 0.18, 1.08; *p* for linear trend = 0.13). The inverse associations between serum levels of specific ω-3 PUFAs and colorectal cancer risk became more significant when cases were restricted to only those followed up for more than 5 years. For men, the odds ratios for Q4 versus Q1 were as follows: 0.10 for α-linoleic acid (95 percent CI: 0.01, 0.86; *p* for linear trend = 0.23), 0.44 for eicosapentaenoic acid (95 percent CI: 0.10, 1.94; *p* for linear trend = 0.13), 0.24 for docosapentaenoic acid (95 percent CI: 0.05, 1.09; *p* for linear trend = 0.07), and 0.07 for docosahexaenoic acid (95 percent CI: 0.01, 0.70; *p* for linear trend = 0.10).

For women, a significantly increased risk for Q3 compared with Q1 was observed for α-linolenic acid (table 4). For the other ω-3 PUFAs, the odds ratios for the highest versus the lowest quartiles were less than 1.0. When those participants who developed colorectal cancer within the first 5 years of follow-up were excluded from the analyses, all ω-3 PUFAs showed a decreased risk at the highest level (data not shown). The odds ratios for Q4 versus Q1 for women were as follows: 0.64 for α-linolenic acid (95 percent CI: 0.11, 3.75; *p* for linear trend = 0.51), 0.55 for eicosapentaenoic acid (95 percent CI: 0.10, 3.11; *p* for linear trend = 0.40), 0.86 for docosapentaenoic acid (95 percent CI: 0.17, 4.45; *p* for linear trend = 0.58), and 0.53 for docosahexaenoic acid (95 percent CI: 0.07, 3.98; *p* for linear trend = 0.80). No significant linear trend was detected between the serum levels of any ω-6 PUFAs and colorectal cancer risk for men or women (table 5). Only eicosadienoic acid showed a significant association with a decreased risk of colorectal cancer at the Q3 versus Q1 level for men (odds ratio = 0.18, 95 percent CI: 0.06, 0.56).

TABLE 4. Associations of serum levels of ω-3 polyunsaturated fatty acids with colorectal cancer risk, by sex, Japan Collaborative Cohort Study for the Evaluation of Cancer Risk, 1988–1997

Q*	Men					Women				
	Value†	Cases (no.)	Controls (no.)	OR*,‡	95% CI*	Value†	Cases (no.)	Controls (no.)	OR‡	95% CI
					α-linolenic acid (18:3 ω-3)					
Q1	<0.69	34	56	1.00		<0.71	14	56	1.00	
Q2	0.69–0.849	13	64	0.22	0.09, 0.55	0.71–0.859	20	63	1.97	0.81, 4.79
Q3	0.85–1.069	14	60	0.25	0.10, 0.59	0.86–1.09	30	59	3.07	1.28, 7.33
Q4	≥1.070	22	61	0.39	0.16, 0.91	≥1.10	22	62	2.19	0.87, 5.47
p trend				0.06					0.15	
					Eicosapentaenoic acid (20:5 ω-3)					
Q1	<1.91	25	60	1.00		<1.73	23	57	1.00	
Q2	1.91–2.719	21	60	0.70	0.33, 1.48	1.73–2.384	20	63	0.56	0.25, 1.26
Q3	2.72–3.839	22	60	0.84	0.38, 1.86	2.385–3.329	20	60	0.67	0.31, 1.48
Q4	≥3.840	15	61	0.44	0.18, 1.08	≥3.330	23	60	0.83	0.39, 1.80
p trend				0.13					0.79	
					Docosapentaenoic acid (22:5 ω-3)					
Q1	<0.68	29	57	1.00		<0.665	27	60	1.00	
Q2	0.68–0.829	14	63	0.36	0.15, 0.86	0.665–0.789	24	56	0.83	0.39, 1.75
Q3	0.83–1.019	24	57	0.55	0.24, 1.24	0.790–0.944	19	64	0.62	0.29, 1.34
Q4	≥1.020	16	64	0.30	0.11, 0.80	≥0.945	16	60	0.64	0.30, 1.39
p trend				0.045					0.14	
					Docosahexaenoic acid (22:6 ω-3)					
Q1	<4.23	22	60	1.00		<4.20	19	59	1.00	
Q2	4.23–5.079	23	59	1.01	0.46, 2.20	4.20–5.094	23	61	1.11	0.47, 2.61
Q3	5.08–6.249	29	61	1.17	0.53, 2.62	5.095–5.919	27	59	1.62	0.72, 3.65
Q4	≥6.25	9	61	0.23	0.07, 0.76	≥5.92	17	61	0.80	0.33, 1.93
p trend				0.07					0.86	

* Q, quartile; OR, odds ratio; CI, confidence interval.

† Values are expressed as the weight percentage of total serum lipids.

‡ Odds ratios were derived from a conditional logistic analysis model adjusted for family history of colorectal cancer in first-degree relatives, body mass index, education, smoking and alcohol drinking history, green leafy vegetable intake, and physical exercise. For men, 83 cases and 241 controls and, for women, 86 cases and 240 controls matched on age and participating institution were involved in the analyses.

DISCUSSION

The main strength of our study is its prospective design and the use of biomarkers to evaluate each fatty acid level. Because we collected serum samples and background data from subjects when they were free of cancer, we were able to eliminate any influence of the cancer itself or recall bias on the results. We confirmed our results by repeating the analyses after excluding persons who developed colorectal cancer within the first 2 or 5 years of follow-up, along with their matched controls, to eliminate any potential effects of undiagnosed colorectal cancer cases at baseline.

Our study design is similar to that of the nested case-control study based on data from the Multiple Risk Factor Intervention Trial (MRFIT) (21). Simon et al. examined 108 cancer cases and 215 controls and found no association between any serum fatty acid component and the risk of fatal cancer. Unfortunately, because of the limited number of subjects, they were unable to estimate risk by organ site. This prospective study is the first known to report an association between colorectal cancer risk and specific serum fatty acids.

We found a marginally significant inverse association between serum level of ω-3 PUFAs and the risk of colorectal cancer in men. The odds ratios for the highest versus lowest quartiles of all ω-3 PUFAs examined were less than 1.0 and were statistically significant ($p < 0.05$), except for eicosapentaenoic acid ($p = 0.13$). These findings support the potential preventive effects of fish oil supplements rich in ω-3 PUFAs against colorectal cancer (22, 23), which have been suggested by a number of clinical studies (3–5, 24, 25). The chemopreventive activity of nonsteroidal antiinflammatory drugs on colorectal tumors has been well documented in a number of experimental studies (26–29). Suppression of cyclooxygenase and inhibition of prostaglandin E_2 synthesis by nonsteroidal antiinflammatory drugs is thought to be the

TABLE 5. Associations of serum levels of ω-6 polyunsaturated fatty acids with colorectal cancer risk, by sex, Japan Collaborative Cohort Study for the Evaluation of Cancer Risk, 1988–1997

Q*	Men					Women				
	Value†	Cases (no.)	Controls (no.)	OR*,‡	95% CI*	Value†	Cases (no.)	Controls (no.)	OR‡	95% CI
					Linoleic acid (18:2 ω-6)					
Q1	<22.94	27	60	1.00		<25.19	19	60	1.00	
Q2	22.94–26.34	19	60	0.80	0.39, 1.60	25.19–27.55	15	60	0.86	0.37, 2.02
Q3	26.35–29.75	19	60	0.66	0.30, 1.43	27.56–30.78	24	60	1.61	0.70, 3.70
Q4	≥29.76	18	61	0.57	0.24, 1.38	≥30.79	28	60	1.88	0.78, 4.52
p trend				0.20					0.12	
					γ-linolenic acid (18:3 ω-6)					
Q1	<0.17	16	59	1.00		<0.21	28	58	1.00	
Q2	0.17–0.269	24	60	1.60	0.74, 3.46	0.21–0.289	20	61	0.60	0.29, 1.26
Q3	0.27–0.359	17	60	0.98	0.44, 2.21	0.29–0.379	18	57	0.53	0.25, 1.13
Q4	≥0.36	26	62	1.99	0.86, 4.62	≥0.380	20	64	0.62	0.30, 1.31
p trend				0.27					0.23	
					Eicosadienoic acid (20:2 ω-6)					
Q1	<0.18	25	52	1.00		<0.17	15	29	1.00	
Q2	0.18–0.199	23	64	0.68	0.33, 1.40	0.17–0.189	20	58	0.69	0.28, 1.72
Q3	0.20–0.219	7	53	0.18	0.06, 0.56	0.19–0.209	22	70	0.52	0.22, 1.22
Q4	≥0.22	28	72	0.71	0.33, 1.53	≥0.21	29	83	0.58	0.25, 1.35
p trend				0.26					0.21	
					Dihomo-γ-linolenic acid (20:3 ω-6)					
Q1	<0.84	17	58	1.00		<0.92	19	59	1.00	
Q2	0.84–1.049	22	59	1.27	0.59, 2.73	0.92–1.079	25	58	1.35	0.62, 2.97
Q3	1.05–1.239	22	62	1.13	0.50, 2.55	1.08–1.349	31	62	1.55	0.76, 3.17
Q4	≥1.24	22	62	1.33	0.60, 2.94	≥1.35	11	61	0.53	0.22, 1.31
p trend				0.55					0.46	
					Arachidonic acid (20:4 ω-6)					
Q1	<3.71	20	59			<4.20	26	60	1.00	
Q2	3.71–4.619	25	61	1.24	0.55, 2.78	4.20–4.879	22	59	0.67	0.31, 1.46
Q3	4.62–5.269	16	59	0.79	0.32, 1.96	4.88–5.634	16	61	0.49	0.22, 1.10
Q4	≥5.27	22	62	1.16	0.49, 2.75	≥5.635	22	60	0.65	0.30, 1.44
p trend				0.99					0.40	
					Docosatetraenoic acid (22:4 ω-6)					
Q1	<0.09	21	56	1.00		<0.085	20	60	1.00	
Q2	0.09–0.099	9	34	0.82	0.31, 2.22	0.085–0.109	27	57	1.47	0.68, 3.15
Q3	0.10–0.119	16	70	0.81	0.35, 1.90	0.110–0.119	13	40	0.87	0.34, 2.21
Q4	≥0.12	37	81	1.58	0.66, 3.78	≥0.12	26	83	0.81	0.35, 1.87
p trend				0.30					0.49	

* Q, quartile; OR, odds ratio; CI, confidence interval.

† Values are expressed as the weight percentage of total serum lipids.

‡ Odds ratios were derived from conditional logistic analysis model adjusted for family history of colorectal cancer in first-degree relatives, body mass index, education, smoking and alcohol drinking history, green leafy vegetable intake, and physical exercise. For men, 83 cases and 241 controls and, for women, 86 cases and 240 controls matched on age and the participating institution were involved in the analyses.

main mechanism for this activity. ω-3 PUFAs are thought to influence the carcinogenic process via their effects on the synthesis of prostaglandins and thromboxanes (1) through a mechanism similar to that of nonsteroidal antiinflammatory drugs. Increased intake of eicosapentaenoic and docosahexaenoic acids might also promote apoptosis in cells of the normal human colonic mucosa (30).

We found no significant association between ω-3 PUFAs and the risk of colorectal cancer in women. α-linolenic and linoleic acid were associated with colorectal cancer inci-

dence in the opposite direction for men and women. MUFAs showed a marginally significant positive association for men only. On the basis of the available data, we cannot suggest a plausible explanation for the gender difference in the association between fatty acids and colorectal cancer risk. Genetic and hormonal factors, nutritional status, and disease are all thought to influence fatty acid metabolism (31). In addition, it has been suggested that female sex hormones play a role in the etiology of colorectal cancer (32, 33). Interestingly, the odds ratios for the highest versus lowest quartiles were less than 1.0 for all of the ω-3 PUFAs examined in women when those women who developed colorectal cancer within the first 5 years of follow-up were excluded. It is possible that physical disorders or medications not evaluated in the present analyses might have influenced the results. Unfortunately, we did not collect data on use of nonsteroidal antiinflammatory drugs and other medications that might have interfered with the association between ω-3 PUFAs and colorectal cancer risk. Further investigation of diet and metabolism will therefore be necessary to clarify these gender interactions. In addition, we did not observe an obvious dose response between serum levels of ω-3 PUFAs and colorectal cancer risk. Additional studies should thus examine whether an optimal level of ω-3 PUFAs is associated with colorectal cancer prevention.

The risks of colorectal cancer are reported to vary by subsite (34, 35). The available data showed no obvious differences between the separate risk of colon cancer and the combined risk of colorectal cancer regarding the association with fatty acids. Although we were unable to estimate the independent risks by subsite in our study because of the limited number of cases, they should be confirmed in future studies with larger sample sizes.

Some limitations that affected interpretation of our results must be noted. First, our subjects were selected from among the participants of a large cohort study. As Kato et al. (36) discussed previously, subjects in cohort studies tend to be homogeneous and health conscious, which might reduce the between-person variation in food consumption and other health-related factors and make detection of associations between individual fatty acids and disease risk more difficult. Moreover, the subjects in the present study were limited to those who donated blood samples: only 36.6 percent of the total cohort. In fact, those who did not donate blood samples were more likely to be highly educated, to consume alcohol daily, and to exercise less compared with those who donated blood samples, regardless of gender. In addition, compared with nonparticipants, male participants tended to be older and female participants tended to be younger. The differences in the background characteristics of the subjects should be considered to generalize our findings. Second, because this was a multicenter study (12), the procedures used to collect blood were not uniform. However, we confirmed that no area had a greatly different distribution of fatty acid levels. In addition, we matched cases and controls by participating institution; therefore, any bias due to differences between areas should have been accounted for.

Third, we used serum samples that were stored at −80°C for 11–14 years to evaluate the levels of fatty acids. Iso et al. (37) examined 31 serum samples taken from subjects in the present cohort in 1990 and again in 1998. They reported an increase in the composition of saturated fatty acids (29.2 percent vs. 30.3 percent) and 20:3 (dihomo-γ linolenic acid) (0.85 percent vs. 0.98 percent), a decline in the composition of MUFAs (22.9 percent vs. 22.4 percent), and no changes in the other fatty acids over this 8-year time interval. Zeleniuch-Jacquotte et al. (11) reported that storage for up to 12 years at −80°C effectively protected PUFAs from oxidation. However, the long-term effects of storage for up to 14 years have not been confirmed. Fourth, although we used the fatty acid composition of serum total lipids as a biomarker, several alternative methods are available for biologic assessment of fat intake. Adipose tissue and the erythrocyte membrane reflect long- and medium-term fatty acid intake, respectively, whereas serum reflects only short-term (weeks to months) intake (10). Although measuring these alternative biomarkers is more expensive and invasive, they might be a better index for use in predicting colorectal cancer. Therefore, our results should be confirmed by using these media. Fifth and finally, we evaluated the fatty acids and background characteristics of the subjects only once, at baseline. These measurements might not accurately reflect the long-term habits of the subjects. Thus, repeated measurements should be considered to reduce measurement errors (11).

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