Biochemical analysis of Temporomandibular Joint (TMJ) Synovial Fluid

Takanori SHIBATA, D.D.Sc., Ph.D.

First Department of Oral & Maxillofacial Surgery, School of Dentistry, Health Sciences University of Hokkaido

INTRODUCTION

Recent improvements in techniques for detecting trace amounts of biologic molecules in small volume of synovial fluid (SF) have lead to analysis on various inflammatory and cartilage degradation markers in the diseased temporomandibular joint(TMJ).

After the last of 1990's, in Japan, a lot of research groups have grown up in this field. They have been focusing and analyzing on various molecules in TMJ synovial fluid , e.g. interleukin 1-β (IL-1β),tumor necrosis factor-α (TNF-α), Interferon-γ , matrix metalloproteinase 1 and 3, tissue inhibitor matrix metalloproteinase 1 and 3 as inflammatory markers , and Chondroitin -4 and 6 sulfates(C4S, C6S), Hyaluronic acid(HA), Keratan sulfate (KS), procollagen Ⅱ C-peptide (pCol Ⅱ-C), pyridinoline and deoxypyrodinoline as cartilage degradation markers.

The purpose of this article is to report on our cross sectional study of the concentrations of the cartilage degradation molecules (HA, C4S, C6S, KS and pCol Ⅱ-C) and the inflammatory molecules(IL-1β , TNF-α) in the direct aspirated SF in patients with TMJ disc disorders, and to discuss their role in the joint pathology.

PATIENTS and METHODS

Patients

All of female patients were diagnosed as a normal disc position (NOR), an anterior displaced disc with (WR) or without reduction (WOR) by MR imagery. All of the joints showed evidence of joint effusion and joint pain.

Joint Fluid

The aspirates of TMJ SF were obtained and treated by the modified direct aspiration method as described previously. The previous study on the exact location of TM joint fluid using MR Imaging in patients with chronic internal derangements revealed that SF in a mouth closed condition was most frequently concentrated in the antero-lateral area of upper compartment of TMJ. This position is just under the articular tubercle and just inside of lateral wall of capsule. Based on this information, the point of needle entry was changed to be just under the articular tubercle. The samples were diluted with physiologic saline (approximately 1.5 g) in vitro. The diluted samples were collected in plastic tubes and then centrifuged at 8000 g for 20 minutes to remove cells and tissue debris. The supernatants were stored in aliquots at - 20° C until used.

Analysis

1) Cartilage degradation molecules

(1)HA, C4S, C6S

CHEMICALS

The standard unsaturated disaccharides, 2-acetamido-2-deoxy-3-0-(β -D-gluco-4- enepyranosyluronic acid)-D-glucose (Δ di-HA), 2-acetamido-2-deoxy-3-0-(β -D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose (Δ di-4S), 2-acetamido-2-deoxy-3-0-(β -D-gluco-4-enepyranosyluronic acid)-6-0-sulfo-D-galactose (Δ di-6S). chondroitinase ABC (EC 4.2.2.4) (CHase ABC), and hyaluronidase derived from Streptococcus dysgalactiae (EC 4.2.2.1) HAase SD) were obtained from Seikagaku Corporation (Tokyo. Japan): 2-cyanoacetamide was purchased from Atdrich(Milwaukee, WI). All other chemicals wcre of reagent gradc. YMC gel PA-120 (YMC Ltd. Kyoto, Japan), packed in a stainless steel column (4.6-mm inner diameter × 250 mm) was used to separate unsaturated disaccharides.

ENZYMATIC DIGESTION OF JOINT FLUIDS

Each joint fluid specimen was diluted 10-fold with distilled water and then digested with CHase ABC and HAase SD as follows. To 200 μL diluted joint fluid, 50 μL CHase ABC solution in distilled water (5 units/ml), 80 μL of 10 mmol/L sodium acetate buffer (pH 8.0), and 70 μL distilled water were added. The

mixture was incubated at 37℃ for 2 hours and then the mixture was ultrafiltered using an Ultrafree C3GC system (molecular size cut-off 10.000; Japan Millipore Ltd, Tokyo, Japan). Because a large mount of HA exists in the sample, and CHase ABC acts more slowly on HA than on CS, HA was not completely reduced to disaccharide by CHase ABC digestion only. Thus, toreduce HA completely to the disaccharide, 30 μL HAase SD solution in distilled water (0.5 units/-ml) and 30 μL of 100 mmol/L sodium acetate buffer (pH6.0) were added to 300 μL of the ultrafiltrate and the mixture was incubated at 37° C for 2 hours. HAase SD derived from Streptococcus reacts with the tetrasaccharides of HA and produces the unsaturated disaccharide of HA (Δ di-HA). After digestion with HAase SD, the mixture was ultrafiltered as previously described, and the filtrate obtained was analyzed by HPLC.

HPLC Analysis

HPLC analysis of the unsaturated disaccharides derived from HA and CS in SF was performed according to the method of Toyoda et al. The HPLC system used was constructed from two pumps (model 880-PU: Japan Spectroscopic Co, Ltd, Tokyo, Japan), an autosampling injector (Model 23 1 ; Gilson, Villiers le Bet, France), a stainless steel column packed with propylaminc-bound silica gel (YMC gel PA- 120). a dry reaction bath (DB-3: Shimamura Instrument Co. Tokyo,Japan), a spectrofluorometer (model 820-FP: Japan Spectroscopic). and an integrator (model 805-GI; Japan Spectroscopic).

The unsaturated disaccharides in each sample were eluted with a gradient of 0 to 100 mmol/L sodium sulfate for 60 minutes at a flow rate of 0.5 mL/min. To the eluent from the column, 100 mmol/L sodium tetraborate buffer (pH 9.0) containing 10 mg/mL 2-cyanoacetamide was added at a flow rate of 0.5 mL/min, and the mixture was passed through a polyetherketone reaction coil (0.8 mm inner dimension × 10 m) set in the dry reaction bath thermostated at 137℃. The effluent was monitored by the spectrofluorometer set at an excitation of 331 nm and emission of 383 nm. The area of each peak corresponding to unsaturated disaccharide was calculated by the integrator. After the concentrations of the Δ di-6S, Δ di-4S, and Δ di-HA were measured by HPLC the ratio of Δ di-6s or Δ di-4S to Δ di-HA, and Δ di-6S to Δ di-4S, were calculated.

(2) KS

KS concentrations was quantified by electrochemilumine-scence immunoassay (ECLIA) with a monoclonal antibody (1-20 5D4). The range of detection level of this assay is 1～500 ng/ml.

(3) pCol Ⅱ-C

pCol Ⅱ-C was quantified by enzyme immunoassay (EIA) kit with a polyclonal antiserum against the bovine propeptide (chondrocalcin test 'TeijinR', Teijin Ltd., Japan). The detection level of this kit is over 0.2 ng/ml.

2) Inflammatory molecules

(1) IL-1β

IL-1β was quantified by enzyme immunoassay (EIA) kit with a blend of monoclonal antibodies against distinct epitopes of IL-1β (MEDGENIX IL-β E ASIATM kit, BioSource Europe S.A. Ltd., Belgium). The detection level of this kit is over 2 pg/ml.

(2) TNF-α

TNF-α was quantified by immunoenzymometric assay kit with a blended of mono-clonal antibodies directd against distinct epitopes of TNF-α are used (TNF- EASIATM BioSource Europe S.A., Belgium). The detection level of this kit is over 3pg/ml.

Statistical analysing method

SAS Ver. 6.0 was used for statistical analysis. Statements in this study indicating a significant difference refer to a p value of 0.05 or less.

RESULTS

1) Cartilage degradation molecules

(1) HA, C4S, C6S

There were no significant differences in the concentration of Δ di-6S, Δ di-4S, and Δ di-HA. among the groups and in the ratio of Δ di-6S or Δ di-4S to Δ di-HA among the groups.

(2) KS

In 62 joints (100%) of 62 joints of 55 female patients (mean 34.1, mode 29 years old), KS in SF was detectable. The mode and ranges of KS concentrations in the WR (24 joints), WOR reduction (24 joints) and OA (14 joints) groups was 1779.4, 617.22 and 387.75, and 280.32～39495.5, 64～8740 and 126.48～3510.5 ng/ml, respectively.This difference among the groups was statistically significant ($p<0.05$).

(3) pCol Ⅱ-C

In 36 joints (73.5%) of 49 joints of 45 female patients (mean 31.8 years), pCol Ⅱ-C in SF was detectable. The frequency of TMJ with detectable pCol Ⅱ-C in the NOR (2 joints), WR (19 joints) and WOR reduction (28 joints) groups was 0%, 57.9% and 89.3%, respectively. This difference among the groups was statistically significant ($p< 0.01$).

2) Inflammatory molecules

(1) IL-1β

In 44 joints (80%) of 55 joints of 49 female patients (mean 34.9 years, mode 27 years), IL-1β in SF was detectable. The frequency of TMJ with detectable IL-β in the WR (19 joints), WOR (21 joints) and OA (15 joints) groups was 100%, 76.2% and 60%, respectively. This difference among the groups was statistically significant ($p< 0.05$).

(2) TNF-α

In 18 joints (32.7%) of 55 joints of 49 female patients (mean 34.9 years), TNF-α in SF was detectable. The frequency of TMJ with detectable TNF-α in the WR (19 joints), WOR (21 joints) and OA (15 joints) groups was 36.8%, 23.8% and 40%. There was no

statistically difference in the detection of TNF-α among the groups.

DISCUSSION and CONCLUSION

1) Cartilage degradation molecules

Although the concentration of C6S, C4S and HA. and the ratio of C6S or C4S to HA in SF dose not reflect the pathosis of patients with disc disorders, analysis of the GAG content in the joint fluid represents a unique way to study joint diseases. Because C6S is the chancteristic CS of cartilaginous tissues, and C4S may be the characteristic CS of synovial tissue, measurement of these molecules, in particular, should provide precise information about alterations in cartilage metabolism and synovial proliferation.

On the other hand, the increased levels of KS in SF may reflect an increased rate of turnover in the extraceller matrix of the TMJ cartilage and degenerative disc in patients with the early stage of the disc disorders, especially with an anterior displaced disc with reduction.The increased levels of pCol Ⅱ-C in SF may reflect an increased rate of synthesis of collagen Ⅱ in the TMJ cartilage of patients with disc disorders, especially with an anterior displaced disc without reduction.

2) Inflammatory molecules

The increased levels of IL-1β in SF may reflect inflammatory changes of the TMJ synovial tissue in the patients with disc disorders, especially with an anterior displaced disc with reduction. But the detection of TNF-α in SF dose not reflect the pathosis of patients with disc disorders.

ACKNOWLEDGMENT

The present studies were supported in part by a Grant-in-Aid for Scientific Research (project No. 07557127, 09470448, 11557155,11771260), Japan Ministry of Education, Science, Sports and Culture.

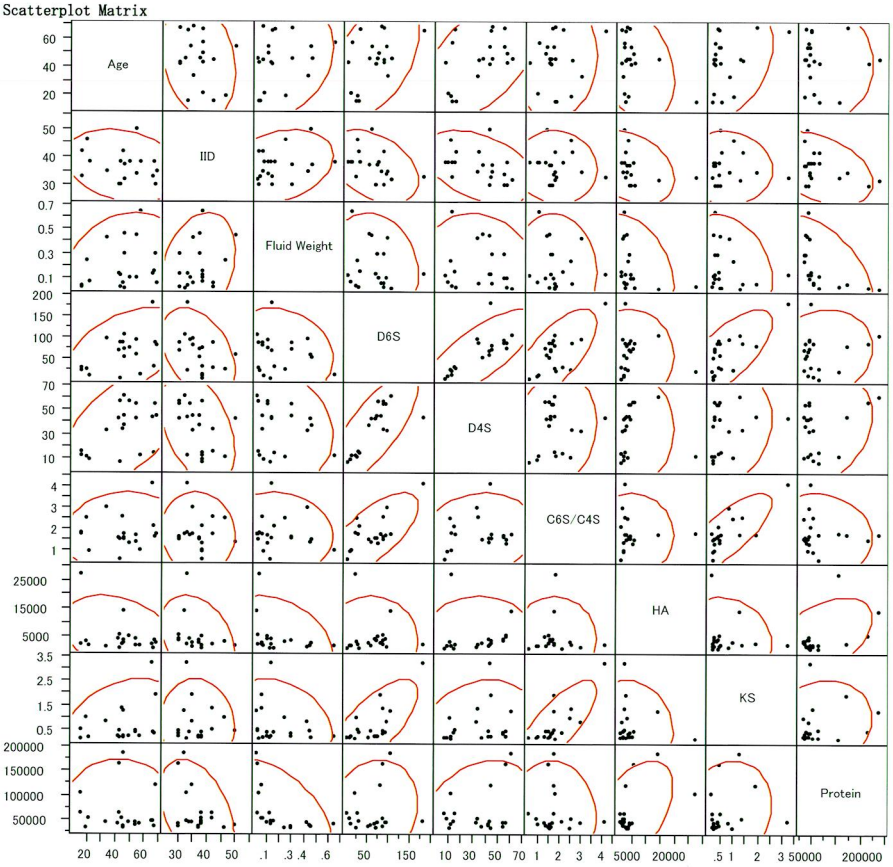
References

T. Shibata, K. Murakami, E. Kubota and H. Maeda: Glycosaminoglycan Components in Temporomandibular Joint Synovial Fluid as Markers of Joint Pathology. J Oral Moxillofac Surg 56 209-213. 1998.

OA
Multivariate
Correlations

	Age	IID	Fluid Weight	D6S	D4S	C6S/C4S	HA	KS	Protein
Age	1.0000	-0.2336	0.2283	0.4758	0.5054	0.0952	-0.3596	0.3216	-0.0526
IID	-0.2336	1.0000	0.2807	-0.3952	-0.4170	-0.0618	-0.3318	-0.0662	-0.4145
Fluid Weight	0.2283	0.2807	1.0000	-0.1408	-0.1239	-0.0873	-0.3505	-0.2244	-0.5283
D6S	0.4758	-0.3952	-0.1408	1.0000	0.7617	0.6906	-0.0433	0.7138	0.1686
D4S	0.5054	-0.4170	-0.1239	0.7617	1.0000	0.1361	0.0263	0.2228	0.3007
C6S/C4S	0.0952	-0.0618	-0.0873	0.6906	0.1361	1.0000	-0.0187	0.7822	-0.0847
HA	-0.3596	-0.3318	-0.3505	-0.0433	0.0263	-0.0187	1.0000	-0.1076	0.5110
KS	0.3216	-0.0662	-0.2244	0.7138	0.2228	0.7822	-0.1076	1.0000	0.1850
Protein	-0.0526	-0.4145	-0.5283	0.1686	0.3007	-0.0847	0.5110	0.1850	1.0000

Scatterplot Matrix



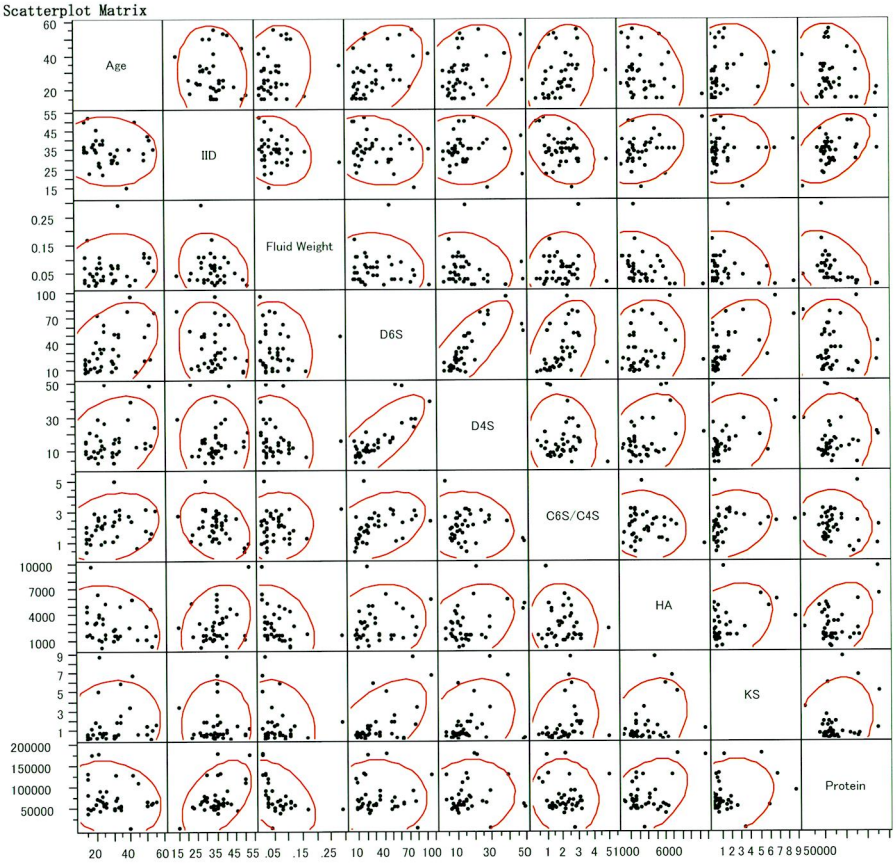
Nonparametric: Spearman's Rho

Variable	by Variable	Spearman Rho	Prob>|Rho|	
IID	Age	-0.1516	0.5234	------
Fluid Weight	Age	0.3273	0.1590	++++++++++++
Fluid Weight	IID	0.2082	0.3785	++++++++
D6S	Age	0.3762	0.1021	+++++++++++++
D6S	IID	-0.4970	0.0258	------------------
D6S	Fluid Weight	-0.1544	0.5158	------
D4S	Age	0.4516	0.0456	++++++++++++++++
D4S	IID	-0.5509	0.0118	--------------------
D4S	Fluid Weight	-0.2425	0.3030	---------
D4S	D6S	0.7759	<0.0001	++++++++++++++++++++++++++++++
C6S/C4S	Age	-0.0498	0.8349	
C6S/C4S	IID	-0.0992	0.6773	--
C6S/C4S	Fluid Weight	-0.1710	0.4710	------
C6S/C4S	D6S	0.4987	0.0252	++++++++++++++++++
C6S/C4S	D4S	0.0391	0.8700	
HA	Age	-0.1236	0.6035	-----
HA	IID	-0.5601	0.0102	--------------------
HA	Fluid Weight	-0.4834	0.0308	-----------------
HA	D6S	0.3308	0.1542	++++++++++++
HA	D4S	0.6451	0.0021	++++++++++++++++++++++++
HA	C6S/C4S	0.0466	0.8452	
KS	Age	0.2314	0.3262	++++++++
KS	IID	0.0357	0.8812	
KS	Fluid Weight	-0.2123	0.3687	---------
KS	D6S	0.6195	0.0036	++++++++++++++++++++++++
KS	D4S	0.2797	0.2323	++++++++++
KS	C6S/C4S	0.6709	0.0012	++++++++++++++++++++++++++
KS	HA	-0.0827	0.7289	--
Protein	Age	-0.1900	0.4224	-------
Protein	IID	-0.2341	0.3206	---------
Protein	Fluid Weight	-0.8358	0.0001	----------------------------------
Protein	D6S	0.1023	0.6679	++++
Protein	D4S	0.0827	0.7289	++
Protein	C6S/C4S	0.1181	0.6200	++++
Protein	HA	0.1353	0.5694	++++
Protein	KS	0.2150	0.3626	++++++++

WOR
Multivariate
Correlations

	Age	IID	Fluid Weight	D6S	D4S	C6S/C4S	HA	KS	Protein
Age	1.0000	-0.0761	0.2134	0.4584	0.3425	0.2473	-0.2229	0.1200	-0.1907
IID	-0.0761	1.0000	-0.2064	-0.2092	-0.0355	-0.3058	0.2800	0.0156	0.4899
Fluid Weight	0.2134	-0.2064	1.0000	-0.0645	-0.1569	0.0733	-0.3111	-0.1742	-0.4456
D6S	0.4584	-0.2092	-0.0645	1.0000	0.7808	0.4097	0.1865	0.5710	-0.0308
D4S	0.3425	-0.0355	-0.1569	0.7808	1.0000	-0.1001	0.3763	0.3242	0.0926
C6S/C4S	0.2473	-0.3058	0.0733	0.4097	-0.1001	1.0000	-0.1157	0.2742	-0.0894
HA	-0.2229	0.2800	-0.3111	0.1865	0.3763	-0.1157	1.0000	0.3589	0.4032
KS	0.1200	0.0156	-0.1742	0.5710	0.3242	0.2742	0.3589	1.0000	0.2184
Protein	-0.1907	0.4899	-0.4456	-0.0308	0.0926	-0.0894	0.4032	0.2184	1.0000

Scatterplot Matrix



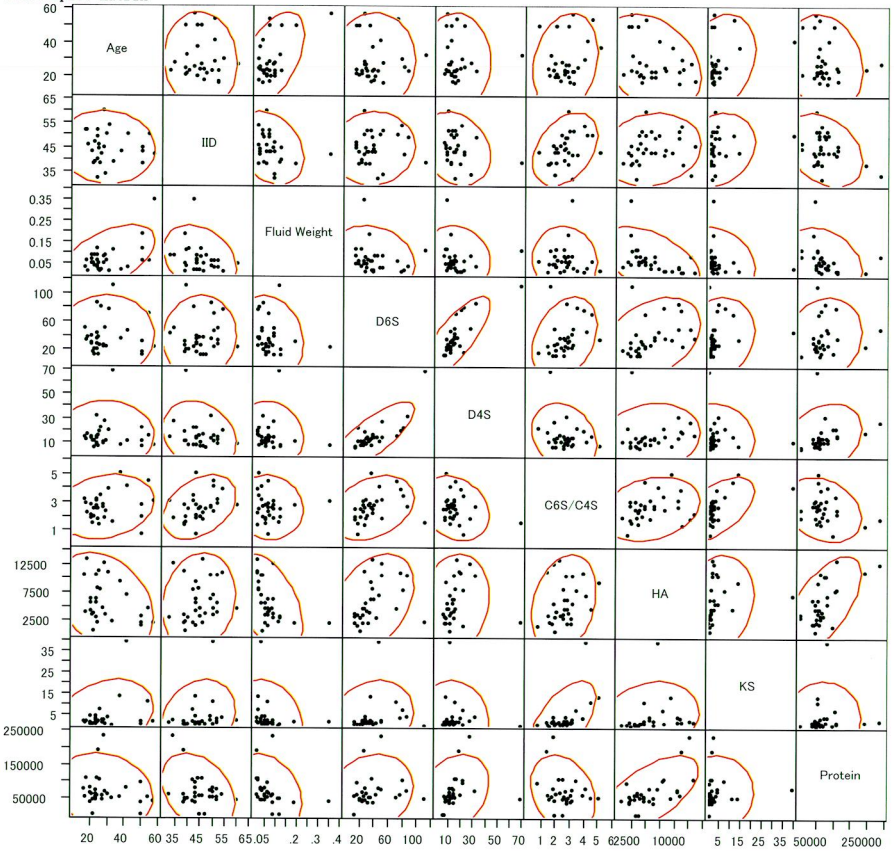
Nonparametric: Spearman's Rho

Variable	by Variable	Spearman Rho	Prob>|Rho|	
IID	Age	-0.1495	0.3705	----
Fluid Weight	Age	0.2674	0.1046	++++++++++
Fluid Weight	IID	-0.2302	0.1644	---------
D6S	Age	0.4845	0.0021	+++++++++++++++++++
D6S	IID	-0.0509	0.7616	--
D6S	Fluid Weight	-0.0677	0.6863	--
D4S	Age	0.3370	0.0386	++++++++++++
D4S	IID	0.1514	0.3643	++++++
D4S	Fluid Weight	-0.1929	0.2459	-------
D4S	D6S	0.7738	<0.0001	+++++++++++++++++++++++++++++++
C6S/C4S	Age	0.3699	0.0223	+++++++++++++++
C6S/C4S	IID	-0.1824	0.2731	-------
C6S/C4S	Fluid Weight	0.0695	0.6783	++
C6S/C4S	D6S	0.6084	<0.0001	++++++++++++++++++++++++
C6S/C4S	D4S	0.0998	0.5510	++
HA	Age	-0.2567	0.1198	----------
HA	IID	0.2487	0.1321	++++++++
HA	Fluid Weight	-0.3883	0.0160	----------------
HA	D6S	0.2369	0.1521	++++++++
HA	D4S	0.1662	0.3186	++++++
HA	C6S/C4S	-0.0194	0.9081	
KS	Age	0.2685	0.1031	++++++++++
KS	IID	0.1060	0.5266	++++
KS	Fluid Weight	-0.1394	0.4038	-----
KS	D6S	0.4337	0.0065	++++++++++++++++
KS	D4S	0.3008	0.0664	++++++++++++
KS	C6S/C4S	0.5049	0.0012	++++++++++++++++++++
KS	HA	0.1722	0.3011	++++++
Protein	Age	-0.1094	0.5131	----
Protein	IID	0.3326	0.0413	++++++++++++
Protein	Fluid Weight	-0.6063	<0.0001	------------------------
Protein	D6S	0.0159	0.9247	
Protein	D4S	0.2626	0.1112	++++++++++
Protein	C6S/C4S	-0.0498	0.7665	
Protein	HA	0.0297	0.8597	
Protein	KS	0.0791	0.6368	++

WR
Multivariate
Correlations

	Age	IID	Fluid Weight	D6S	D4S	C6S/C4S	HA	KS	Protein
Age	1.0000	-0.0605	0.5482	0.0278	0.0056	0.1998	-0.3100	0.2812	-0.1721
IID	-0.0605	1.0000	-0.2951	0.0520	-0.2180	0.3801	0.1474	0.2097	-0.3310
Fluid Weight	0.5482	-0.2951	1.0000	-0.1648	-0.0530	-0.0557	-0.4867	-0.2046	-0.2944
D6S	0.0278	0.0520	-0.1648	1.0000	0.7848	0.3253	0.3729	0.2009	0.1629
D4S	0.0056	-0.2180	-0.0530	0.7848	1.0000	-0.2468	0.1420	-0.0826	0.2745
C6S/C4S	0.1998	0.3801	-0.0557	0.3253	-0.2468	1.0000	0.2574	0.5694	-0.2598
HA	-0.3100	0.1474	-0.4867	0.3729	0.1420	0.2574	1.0000	0.1972	0.6295
KS	0.2812	0.2097	-0.2046	0.2009	-0.0826	0.5694	0.1972	1.0000	0.0505
Protein	-0.1721	-0.3310	-0.2944	0.1629	0.2745	-0.2598	0.6295	0.0505	1.0000

Scatterplot Matrix



Nonparametric: Spearman's Rho

Variable	by Variable	Spearman Rho	Prob>|Rho|	
IID	Age	-0.0789	0.6732	--
Fluid Weight	Age	0.2271	0.2191	+++++++
Fluid Weight	IID	-0.3453	0.0571	----------
D6S	Age	0.0245	0.8960	
D6S	IID	0.0909	0.6269	++
D6S	Fluid Weight	-0.2556	0.1651	----------
D4S	Age	-0.0378	0.8399	
D4S	IID	-0.1455	0.4348	-----
D4S	Fluid Weight	-0.2674	0.1459	----------
D4S	D6S	0.7214	<0.0001	+++++++++++++++++++++++++++
C6S/C4S	Age	0.1524	0.4132	++++++
C6S/C4S	IID	0.4379	0.0137	+++++++++++++++
C6S/C4S	Fluid Weight	-0.1641	0.3778	------
C6S/C4S	D6S	0.4881	0.0053	++++++++++++++++
C6S/C4S	D4S	-0.1117	0.5495	-----
HA	Age	-0.2178	0.2391	---------
HA	IID	0.2849	0.1203	++++++++++
HA	Fluid Weight	-0.6386	<0.0001	----------
HA	D6S	0.5851	0.0005	++++++++++++++++++++
HA	D4S	0.4379	0.0137	+++++++++++++++
HA	C6S/C4S	0.2828	0.1232	++++++++++
KS	Age	0.1873	0.3131	++++++
KS	IID	0.3497	0.0538	++++++++++++
KS	Fluid Weight	-0.4985	0.0043	----------
KS	D6S	0.4935	0.0048	++++++++++++++++
KS	D4S	0.1480	0.4269	++++
KS	C6S/C4S	0.5954	0.0004	+++++++++++++++++++++
KS	HA	0.5782	0.0007	+++++++++++++++++++++
Protein	Age	-0.1384	0.4579	-----
Protein	IID	-0.0907	0.6276	--
Protein	Fluid Weight	-0.5595	0.0011	----------
Protein	D6S	0.3583	0.0478	++++++++++++++
Protein	D4S	0.6411	<0.0001	++++++++++++++++++++++++
Protein	C6S/C4S	-0.2066	0.2649	---------
Protein	HA	0.5568	0.0011	+++++++++++++++++++++
Protein	KS	0.3099	0.0897	++++++++++++