Science Translational Medicine
AAAS

www.sciencetranslationalmedicine.org/cgi/content/full/8/346/346ra90/DC1

Supplementary Materials for

Radiocarbon dating reveals minimal collagen turnover in both healthy and osteoarthritic human cartilage

Katja M. Heinemeier, Peter Schjerling, Jan Heinemeier, Mathias B. Møller, Michael R. Krogsgaard, Tomas Grum-Schwensen, Michael M. Petersen, Michael Kjaer*

*Corresponding author. Email: michaelkjaer@sund.ku.dk

Published 6 July 2016, *Sci. Transl. Med.* **8**, 346ra90 (2016)
DOI: 10.1126/scitranslmed.aad8335

This PDF file includes:

Supplementary Materials

SUPPLEMENTARY METHODS

Histology

Serial cryo-sections (10 µm) were cut from the edge of the embedded samples facing the two punch biopsies (to obtain histological scores that best represented the tissue obtained with punch biopsies) (Fig. 1). For each sample two sections were stained with H&E and two with Safranin-O and Fast Green. Prior to histological grading, the section with the best technical quality was chosen. The sections were graded according to a modified version of the Mankin Histologic-Histochemical Grading System (*25*). The system consists of 4 parameters; structure (0-6), cells (0-3), Safranin-O staining (0-4), and tidemark integrity (0-1). Owing to the way the specimens were chosen and obtained, tidemark integrity could not be evaluated (as the underlying bone was not included) and structure grades of 5 and 6 could not be achieved (as areas with severe cartilage loss had to be avoided in order to obtain sufficient tissue for analyses). Therefore the severity of cartilage damage was graded from 0 to 11, where higher numbers represent more severe damage. One blinded observer evaluated the slides. The figures and descriptions in (*35*) were used as a reference.

Hydroxyproline, GAG and DNA measurement

For hydroxyproline measurement 50 µl of the papain digest was hydrolyzed in 950 µl of 6-M HCl at 110°C for 24 h. Hydrolyzates were dried, rehydrated with distilled water, and dried again. The hydrolyzates were resuspended and diluted appropriately in acetate–citrate buffer (0.6% acetic acid, 130 mM citric acid, 440 mM sodium acetate, 425 mM NaOH, pH 6.0). One hundred fifty µl diluted sample was mixed with 75 µl of chloramine-T solution (60 mM chloramine-T, 50% 1-propanol) and incubated at room temp for 20 min. Seventy-five µl aldehyde perchloric acid solution [1 M 4-dimethylaminobenzaldehyde, 60% 1-propanol, 22% perchloric acid (70–72%)] was added to

samples and they were incubated at 60°C for 25 min. Optical density was read at 570 nm and samples were compared to a standard curve made from known concentrations of hydroxyproline (Sigma, H1637) to determine hydroxyproline concentration. Hydroxyproline concentrations were converted to collagen concentration by multiplying by 7.5 (*36*).

For GAG quantification, 10 µl of appropriately diluted papain digest was mixed with 190 µl 1,9-dimethylmethylene blue (DMB) (Sigma 341088) solution (38 µM DMB in 40 mM NaCl, 40 mM glycine, pH 3), and absorbance was read at 540 and 595 nm (the reading at 595 nm was subtracted from the reading at 540 nm). Samples values were compared to a standard curve made with known amounts of chondroitin sulfate C (Sigma C4384).

DNA content was quantified by real time PCR. A 50x dilution of the papain digest was amplified with primers for the myogenin promoter (Sense: AGGTGCTGTCAGGAAGCAAGGA, anti-sense: TAGGGGGAGGAGGGAACAAGGA) in a 25 µl SYBR Green PCR reaction containing 1×Quantitect SYBR Green Master Mix (Qiagen) and 200 nM of each primer. The PCR amplification was monitored real-time using the MX3005P Real-time PCR machine (Stratagene). The C_t values were related to a standard curve made with known concentrations of DNA oligos (Ultramer oligos, Integrated DNA Technologies, Inc.) with a DNA sequence corresponding to the sequence of the expected PCR product. Based on these C_t values, and accounting for the PCR efficiency, the quantity of DNA molecules was determined. This number was normalized with the tissue dry weight. The specificity of the PCR products was confirmed by comparing the melt curves for the unknown samples with melt curves of the DNA oligos.

Isotope analyses

For all subjects samples of purified cartilage collagen from both highly loaded and moderately loaded areas of the tibial plateau were analyzed for ^{14}C and stable isotopes (^{13}C and ^{15}N). Further,

from a subgroup of subjects, raw (non-purified) cartilage samples (subjects 1, 3, 5, 10, 13, & 15), as well as isolated GAG fractions (subjects 6, 7, 19, & 22) were analyzed for ^{14}C and stable isotopes. Samples were analyzed at the AMS ^{14}C Dating Centre, Aarhus University (Denmark). Samples for accelerator mass spectrometry (AMS) were combusted with CuO in sealed combustion tubes at 950 °C, and the resulting CO_2 was submitted for graphitization and AMS analysis at the Accelerator Mass Spectrometry Lab, Accium Biosciences under the direction of U. Zoppi (*37*). The radiocarbon dating results are reported according to international convention (*38*) and ^{14}C content are given as pMC (percent modern carbon) based on the measured $^{14}C/^{13}C$ ratio corrected for the natural isotopic fractionation by normalizing the result to the standard $\delta^{13}C$ value of –25‰ VPDB [Vienna Pee Dee Belemnite ($\delta^{13}C$ calibration standard)]. Stable isotope values of $\delta^{13}C$, $\delta^{15}N$, carbon and nitrogen fraction (by dry weight), and carbon/nitrogen (C/N) atomic ratios were measured at the Aarhus AMS Centre by continuous-flow mass spectrometry.

SUPPLEMENTARY TABLES

Table S1-A: ^{14}C concentration, stable isotope data and Mankin scores for all healthy tissue samples

Individual data for donors of healthy tibial plateau cartilage, including ^{14}C concentration (pMC = percent modern carbon), and stable isotope data ($\delta^{13}C$ and $\delta^{15}N$) (se details in methods and materials). The bomb pulse calibration year(s) represents the year or years (after donor birth) at which the atmospheric level corresponded to the measured 14C concentration (pMC) of the tissue sample. For most donors born before or in the beginning of the bomb pulse peak, two possible corresponding years are given, which represent the ascending and descending part of the bomb-pulse curve.

Tissue fractions other than collagen are highlighted. Healthy cartilage was obtained from osteosarcoma patients who have undergone joint surgery due to disease in the femur. None of these donors had disease involving the tibia (see details in methods and materials).

Subject ID	AMS lab no AAR-	Cartilage state	Sex	Birth year	Load/location	Mankin score	Tissue fraction	^{14}C level (pMC)	Bomb-pulse calibration year	$\delta^{13}C$ (VPDB)	$\delta^{15}N$ (air)	Carbon (weight %)	Nitrogen (weight %)	C/N ratio (atomic)
1	19090	Healthy	F	1947	Moderate load/peripheral	2	**Raw cartilage**	112.42±0.29	1957/1995	-19.61±0.29	13.05±0.30	44.55±0.01	14.40±0.01	3.61±0.01
1	19091	Healthy	F	1947	Moderate load/peripheral	2	Extracted collagen	114.14±0.29	1958/1991	-20.21±0.21	12.37±0.21	48.78±0.01	15.80±0.01	3.60±0.01
1	19092	Healthy	F	1947	High load/central	2	**Raw cartilage**	108.27±0.28	1957/2000	-20.47±0.29	13.53±0.30	37.21±0.01	12.56±0.01	3.46±0.01
1	19093	Healthy	F	1947	High load/central	2	Extracted collagen	105.87±0.31	a	-18.70±0.29	13.50±0.30	44.94±0.01	15.68±0.01	3.34±0.01
2	20202/21462	Healthy	M	1948	Moderate load/peripheral	0	Extracted collagen	121.23±0.38	1960/1984	-20.56±0.36	11.65±0.42	47.40±0.01	15.86±0.01	3.49±0.01
2	20203/21463	Healthy	M	1948	High load/central	5	Extracted collagen	112.61±0.35	1957/1995	-19.54±0.36	12.96±0.42	47.23±0.01	16.07±0.01	3.43±0.01
3	19086	Healthy	F	1953	Moderate load/peripheral	1	**Raw cartilage**	119.79±0.31	1961/1985	-21.35±0.21	12.62±0.21	43.39±0.01	12.20±0.01	4.15±0.01
3	19087	Healthy	F	1953	Moderate load/peripheral	1	Extracted collagen	128.39±0.34	1960/1980	-18.78±0.29	14.77±0.30	27.31±0.01	9.09±0.01	3.51±0.01
3	19088	Healthy	F	1953	High load/central	2	**Raw cartilage**	115.92±0.30	1958/1991	-19.51±0.29	13.87±0.30	45.74±0.01	15.49±0.01	3.45±0.01
3	19089	Healthy	F	1953	High load/central	2	Extracted collagen	117.09±0.35	1958/1989	-19.47±0.29	13.31±0.30	45.77±0.01	15.22±0.01	3.51±0.01
4	20220/21480	Healthy	M	1954	Moderate load/peripheral	1	Extracted collagen	151.31±0.47	1962/1971	-19.26±0.19	12.29±0.32	44.24±0.01	15.30±0.01	3.37±0.01
4	20221/21481	Healthy	M	1954	High load/central	2	Extracted collagen	147.79±0.46	1962/1971	-20.36±0.19	13.22±0.32	44.02±0.01	15.44±0.01	3.33±0.01
5	19102	Healthy	F	1957	Moderate load/peripheral	1	**Raw cartilage**	132.93±0.34	1961/1978	-20.41±0.29	13.11±0.30	43.05±0.01	13.16±0.01	3.82±0.01
5	19103	Healthy	F	1957	Moderate load/peripheral	1	Extracted collagen	144.63±0.38	1962/1972	-20.38±0.21	14.06±0.21	38.94±0.01	12.23±0.01	3.75±0.01
5	19104	Healthy	F	1957	High load/central	1	**Raw cartilage**	126.91±0.33	1960/1980	-20.00±0.29	12.02±0.30	42.31±0.01	12.99±0.01	3.80±0.01
5	19105	Healthy	F	1957	High load/central	1	Extracted collagen	137.75±0.38	1962/1975	-19.56±0.29	11.84±0.30	45.16±0.01	15.26±0.01	3.45±0.01
6	20204/21464	Healthy	M	1971	Moderate load/peripheral	0	Extracted collagen	123.85±0.39	1982	-20.02±0.17	12.22±0.26	45.39±0.01	15.60±0.01	3.40±0.01
6	20205/21465	Healthy	M	1971	High load/central	4	Extracted collagen	129.78±0.40	1979	-20.16±0.19	12.03±0.32	46.14±0.01	15.36±0.01	3.51±0.01
6	21448	Healthy	M	1971	Moderate load/peripheral	-	**Extracted GAGs**	103.35±0.32	a	-27.17±0.35	b	9.27±0.01	1.46±0.01	7.40±0.01
6	21449	Healthy	M	1971	High load/central	-	**Extracted GAGs**	102.34±0.35	a	-32.47±0.35	b	8.39±0.01	1.61±0.01	6.10±0.01
7	20206/21466	Healthy	M	1971	Moderate load/peripheral	0	Extracted collagen	121.11±0.38	1985	-19.57±0.19	11.60±0.32	45.36±0.01	14.82±0.01	3.57±0.01
7	20207/21467	Healthy	M	1971	High load/central	2	Extracted collagen	128.57±0.40	1980	-19.61±0.19	10.81±0.32	44.23±0.01	14.97±0.01	3.45±0.01
7	21446	Healthy	M	1971	Moderate load/peripheral	-	**Extracted GAGs**	102.74±0.32	a	-32.53±0.35	b	6.30±0.01	0.67±0.01	10.90±0.01
7	21447	Healthy	M	1971	High load/central	-	**Extracted GAGs**	104.24±0.32	a	-25.89±0.25	b	12.68±0.01	2.40±0.01	6.19±0.01
8	20222/21482	Healthy	M	1997	Moderate load/peripheral	2	Extracted collagen	105.24±0.34	a	-20.71±0.42	10.29±0.41	43.32±0.01	14.18±0.01	3.56±0.01
8	-/21483	Healthy	M	1997	High load/central	1	Extracted collagen	107.77±0.35	a	Sample lost	Sample lost	Sample lost	Sample lost	Sample lost

[a] For very low pMC values (< 108) close to present day no meaningful calibration can be made due to the low slope of the bomb pulse curve in recent time.

[b] Too low nitrogen content for measurement

Table S1-B - ^{14}C concentration, stable isotope data and Mankin scores for all OA tissue samples

Individual data for osteoarthritic donors of tibial plateau cartilage. Cartilage was obtained from knee arthroplasty operations due to primary OA. See table S1-A for caption

Subject ID	AMS lab no AAR-	Cartilage state	Sex	Birth year	Load/location	Mankin score	Tissue fraction	^{14}C level (pMC)	Bomb-pulse calibration year	$\delta^{13}C$ (VPDB)	$\delta^{15}N$ (air)	Carbon (weight %)	Nitrogen (weight %)	C/N ratio (atomic)
9	20216/21476	OA	F	1935	Moderate load/peripheral	0	Extracted collagen	102.95±0.31	a	-20.48±0.19	13.02±0.32	45.51±0.01	14.49±0.01	3.66±0.01
9	20217/21477	OA	F	1935	High load/central	5	Extracted collagen	102.14±0.33	a	-21.93±0.19	12.59±0.32	49.13±0.01	12.58±0.01	4.56±0.01
10	19098	OA	F	1944	Moderate load/peripheral	3	**Raw cartilage**	105.14±0.28	a	-24.73±0.29	No value	42.06±0.01	No value	No value
10	19099	OA	F	1944	Moderate load/peripheral	3	Extracted collagen	103.88±0.26	a	-19.37±0.21	11.86±0.21	43.98±0.01	14.72±0.01	3.49±0.01
10	19100	OA	F	1944	High load/central	6	**Raw cartilage**	102.92±0.26	a	-20.34±0.29	9.99±0.30	41.51±0.01	12.69±0.01	3.82±0.01
10	19101	OA	F	1944	High load/central	6	Extracted collagen	100.54±0.28	a	-19.42±0.29	9.50±0.30	45.12±0.01	15.24±0.01	3.45±0.01
11	20218/21478	OA	M	1950	Moderate load/peripheral	2	Extracted collagen	149.66±0.47	1962/1971	-20.78±0.19	12.52±0.32	48.85±0.01	13.43±0.01	4.24±0.01
11	20219/21479	OA	M	1950	High load/central	9	Extracted collagen	117.46±0.37	1960/1988	-20.33±0.11	14.17±0.19	45.75±0.01	14.08±0.01	3.79±0.01
12	20212/21472	OA	F	1955	Moderate load/peripheral	1	Extracted collagen	137.32±0.43	1962/1975	-19.99±0.11	10.27±0.19	44.29±0.01	15.10±0.01	3.42±0.01
12	20213/21473	OA	F	1955	High load/central	7	Extracted collagen	147.27±0.46	1962/1972	-20.02±0.19	12.23±0.32	43.64±0.01	14.25±0.01	3.57±0.01
13	19094	OA	F	1958	Moderate load/peripheral	1	**Raw cartilage**	125.15±0.32	1961/1981	-20.77±0.29	12.74±0.30	39.62±0.01	12.11±0.01	3.82±0.01
13	19095	OA	F	1958	Moderate load/peripheral	1	Extracted collagen	137.10±0.36	1960/1976	-20.60±0.29	12.28±0.30	44.90±0.01	15.25±0.01	3.44±0.01
13	19096	OA	F	1958	High load/central	8	**Raw cartilage**	117.23±0.37	1960/1988	-21.75±0.29	12.25±0.30	33.93±0.01	9.95±0.01	3.98±0.01
13	19097	OA	F	1958	High load/central	8	Extracted collagen	132.10±0.37	1960/1978	-20.38±0.21	12.84±0.21	39.04±0.01	13.09±0.01	3.50±0.01
14	20214/21474	OA	M	1961	Moderate load/peripheral	0	Extracted collagen	138.51±0.43	1962/1975	-19.36±0.19	11.93±0.32	44.38±0.01	14.47±0.01	3.58±0.01
14	20215/21475	OA	M	1961	High load/central	4	Extracted collagen	136.92±0.34	1960/1976	-20.20±0.19	11.74±0.32	46.76±0.01	14.57±0.01	3.74±0.01
15	19106	OA	F	1963	Moderate load/peripheral	1	**Raw cartilage**	126.61±0.34	1980	-21.25±0.21	12.44±0.21	42.36±0.01	13.04±0.01	3.79±0.01
15	19107	OA	F	1963	Moderate load/peripheral	1	Extracted collagen	132.41±0.42	1978	-19.92±0.29	12.85±0.30	45.39±0.01	15.27±0.01	3.47±0.01
15	19108	OA	F	1963	High load/central	1	**Raw cartilage**	120.51±0.30	1985	-21.04±0.29	11.89±0.30	42.07±0.01	12.71±0.01	3.86±0.01
15	19109	OA	F	1963	High load/central	1	Extracted collagen	130.87±0.33	1979	-18.90±0.29	13.08±0.30	44.68±0.01	15.26±0.01	3.42±0.01
16	20208/21468	OA	F	1965	Moderate load/peripheral	0	Extracted collagen	134.92±0.42	1977	-19.87±0.11	11.81±0.19	44.32±0.01	14.57±0.01	3.55±0.01
16	20209/21469	OA	F	1965	High load/central	1	Extracted collagen	135.54±0.42	1976	-20.22±0.19	11.88±0.32	29.21±0.01	8.89±0.01	3.83±0.01
17	20210/21470	OA	F	1967	Moderate load/peripheral	1	Extracted collagen	127.80±0.40	1980	-20.09±0.19	11.83±0.32	44.90±0.01	14.53±0.01	3.61±0.01
17	20211/21471	OA	F	1967	High load/central	-	Extracted collagen	140.09±0.44	1974	-19.47±0.19	11.75±0.32	30.13±0.01	9.72±0.01	3.62±0.01
18	20198/21459	OA	M	1968	Moderate load/peripheral	2	Extracted collagen	121.40±0.38	1984	-19.77±0.13	10.19±0.21	46.29±0.01	16.07±0.01	3.36±0.01
19	20192/21453	OA	F	1969	Moderate load/peripheral	0	Extracted collagen	124.55±0.39	1982	-19.22±0.17	11.71±0.26	46.27±0.01	15.83±0.01	3.41±0.01
19	20193/21454	OA	F	1969	High load/central	3	Extracted collagen	129.03±0.40	1980	-19.24±0.36	12.89±0.42	47.87±0.01	15.14±0.01	3.69±0.01
19	21442	OA	F	1969	Moderate load/peripheral	-	**Extracted GAGs**	103.54±0.32	a	-29.86±0.25	b	7.33±0.01	0.95±0.01	9.00±0.01
19	21443	OA	F	1969	High load/central	-	**Extracted GAGs**	104.02±0.32	a	-26.51±0.35	b	18.07±0.01	3.49±0.01	6.05±0.01
20	20194/21455	OA	M	1970	Moderate load/peripheral	0	Extracted collagen	118.77±0.37	1986	-19.51±0.36	12.75±0.42	45.89±0.01	16.07±0.01	3.33±0.01
20	20195/21456	OA	M	1970	High load/central	2	Extracted collagen	125.99±0.39	1981	-18.56±0.36	10.85±0.42	45.88±0.01	15.95±0.01	3.36±0.01
21	20189/21450	OA	M	1971	Moderate load/peripheral	0	Extracted collagen	118.32±0.37	1987	-19.63±0.17	10.82±0.26	45.43±0.01	15.58±0.01	3.41±0.01
21	20190/21451	OA	M	1971	High load/central	1	Extracted collagen	120.25±0.37	1985	-20.10±0.36	13.21±0.42	47.23±0.01	16.09±0.01	3.43±0.01
21	20191/21452	OA	M	1971	High load/central	3	Extracted collagen	118.70±0.37	1986	-18.89±0.36	11.32±0.42	46.44±0.01	16.18±0.01	3.35±0.01
22	20200/21460	OA	F	1972	Moderate load/peripheral	1	Extracted collagen	120.37±0.37	1985	-20.53±0.13	13.07±0.21	47.12±0.01	15.41±0.01	3.57±0.01
22	20201/21461	OA	F	1972	High load/central	3	Extracted collagen	124.76±0.39	1982	-20.29±0.36	13.63±0.42	46.91±0.01	15.26±0.01	3.59±0.01
22	21444	OA	F	1972	Moderate load/peripheral	-	**Extracted GAGs**	100.56±0.31	a	-31.66±0.35	b	5.56±0.01	0.99±0.01	6.56±0.01
22	21445	OA	F	1972	High load/central	-	**Extracted GAGs**	102.16±0.34	a	-29.28±0.35	b	6.65±0.01	1.22±0.01	6.38±0.01
23	20196/21457	OA	F	1973	Moderate load/peripheral	1	Extracted collagen	121.73±0.38	1985	-18.07±0.36	10.13±0.42	45.89±0.01	15.84±0.01	3.38±0.01
23	20197/21458	OA	F	1973	High load/central	6	Extracted collagen	124.33±0.39	1982	-19.92±0.36	10.37±0.42	45.65±0.01	15.82±0.01	3.37±0.01

[a]For very low pMC values (< 108) close to present day no meaningful calibration can be made due to the low slope of the bomb pulse curve in recent time.

[b]Too low nitrogen content for measurement