Past, present and future methods for assessment of chondrocytes' viability: A new parameter for determination of the postmortem interval?

Armin Alibegović

Institute of forensic medicine

University *of* *Ljubljana*
Faculty *of* *Medicine*

Refereed papers

Drobnič M, Marš T, Alibegović A, Bole V, Balažic J, Grubič Z, Brecelj J. Viability of human chondrocytes in an ex vivo model in relation to temperature and cartilage depth. Folia Biol (Praha) 2005; 51:103-8.

Maličev E, Kregar-Velikonja N, Barlič A, Alibegović A, Drobnič M. Comparison of articular and auricular cartilage as a cell source for the autologous chondrocyte implantation. J Orthop Res 2009; 27(7):943-8. doi:10.1002/jor.20833

Paper presentations

2004-09-20 The influence of time and temperature on the post-mortem viability of human chondrocytes in an ex-vivo knee cartilage model. 9th International conference on life sciences of Slovenia. Nova Gorica, Slovenia.

2007-09-29 The acute injury of the surgical sutures on the human articular cartilage. 7th World congress of the international cartilage repair society ICRS. Warsaw, Poland.

2007-10-02 Human auricular cartilage - an alternative source of chondrocytes for autologous chondrocyte implantation (ACI). 7th World congress of the international cartilage repair society ICRS. Warsaw, Poland.

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- studies longer then expected
- chondrocytes lived several weeks

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determination of the postmortem interval (PMI)
one of the most important questions in the forensic medicine

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Alibegović A, Petrovič D, Kregar-Velikonja N, Balažic J, Drobnič M. Comparison of cartilage sources and methods for the ex-vivo determination of chondrocyte viability. Rom J Leg Med 2011; 19(4):265-70. doi:10.4323/rjlm.2011.265

Alibegović A, Balažic J, Petrovič D, Kregar-Velikonja N, Blagus R, Šuput D, Drobnič M. The optimal combination of cartilage source and apparatus for long-term in vitro chondrocyte viability analysis. J Forensic Sci. 2012; 57:1601-7. doi:10.1111/j.1556-4029.2012.02175.x

Alibegović A, Balažic J, Petrovič D, Hribar G, Blagus R, Drobnič M. Viability of human articular chondrocytes harvested post-mortem: changes with time and temperature of in vitro culture conditions. J Forensic Sci. 2014; 58(3):325-32. doi:10.1111/1556-4029.12330

Alibegović A. Cartilage: a new parameter for the determination of the postmortem interval?. J Forensic Leg Med. (in press). doi:http://dx.doi.org/10.1016/j.jflm.2014.08.005

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2007-10-02 Human auricular cartilage - an alternative source of chondrocytes for autologous chondrocyte implantation (ACI). 7th World congress of the international cartilage repair society ICRS. Warsaw, Poland.

2010-05-13 Instrumental analysis of cartilage in forensic medicine. 19th International meeting on forensic medicine Alpe - Adria – Panonia. Tavagnacco-Udine, Italy. (oral communication)

2010-09-27 The optimal temperature for the long term ex/vivo cartilage storage : 11°C is superior to 4°C, 23°C, and 35°C. 9th World congress of the international cartilage repair society. Barcelona, Spain. (oral communication)

2011-06-24 Comparison of cartilage sources and methods for the ex-vivo determination of chondrocyte viability. 4th Symposium of the Osteuropa-Verein. Tirgu Mures, Romania. (oral communication)

2011-06-01 Estimation of the post-mortem time interval according to the articular chondrocyte viability curves at different temperature levels in vitro. 20th International Meeting on Forensic Medicine Alpe-Adria-Pannonia. 2011 Jun 1-4. Bratislava, Slovakia. (poster)

2011-07-05 Cartilage as the tissue for determination of postmortem interval. International Symposium of Sports Medicine. Las Palmas, Spain. (oral communication)

2013-06-07 CVA or CLSM for estimation of PMI?. 22nd International Meeting on Forensic Medicine Alpe-Adria-Pannonia. Kraków, Poland. (oral communication)

- doubts and conclusions

- cartilage as a compartment
- determination of PMI

- particular attributes of cartilage - support this idea

Accepted Manuscript

Cartilage: A new parameter for the determination of the postmortem interval?

Armin Alibegović, MD, PhD, Specialist in Forensic and Legal Medicine

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Substrate	Attributes
Articular cartilage	distance from the torso – the source of the saprophytic microorganisms tissue without vessels and nerves
Ground substance	semi-liquid gel
- Extracellular matrix	tenacious and dense but porous and permeable matrix pores with 6 nm average diameter obstruct the spread of microorganisms highly hydrophilic
- Water	60 to 78 % of the net weight of the hyaline cartilage storage for gases, nutrients, proteins, lipids, electrolytes diffusion of solutes (gases, nutrients, metabolic waste, ...)
Chondrocytes	homogeneous cell population a small number of cells in the tissue (10 % of the tissue volume) modest requirements for oxygen and nutrients low cellular activity, proliferation exceptionally functioning in an environment with a lower pH

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anatomical, morphological, mechanical, physical and chemical properties of cartilage, in particular of the articular cartilage, are those with which the cartilage could be determined as a compartment

Could cartilage be used for the determination of PMI?

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- small number of chondrocytes - modest requirements for nutrients and oxygen
- abundant ECM with different solutes - diffuse to the chondrocytes after clinical death

- allow the survival of the chondrocytes several days after the individual's death

- ECM provide an extra protection to the chondrocytes against microorganisms - spread postmortem

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- researches on the chondrocytes long-term survival
- mainly chondrocytes' cultivation or conservation
- for clinical use - especially for articular cartilage transplantation

- observation of the difference in the proportion of chondrocytes
- survived during the time and at different temperatures

- human allografts kept in the tissue banks at 4 °C and under optimal conditions
- ~ 70 % of the chondrocytes survived for one month
- ~ 35 % of the chondrocytes survived for two months

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gradual decline in the proportion of chondrocytes that survive clearly demonstrated that chondrocytes viability was a function of time and temperature, which could be useful for the determination of PMI

Past methods

Rom J Leg Med [19] 265-270 [2011]
DOI: 10.4323/rjlm.2011.265

Comparison of cartilage sources and methods for the ex-vivo determination of chondrocyte viability

Armin Alibegović[1], Danijel Petrovič[2], Nevenka Kregar Velikonja[3], Jože Balažic[4], Matej Drobnič[5]*

- aim - best combination of sampling source and measuring method

- one donor

- three sources of cartilage biopsy
    - knee
    - ankle
    - ear

- three chondrocyte viability protocols
    - manual counting under a microscope (MCM)
    - cell viability analyzer (CVA)
    - flow cytometer (FCM)

- two storage temperatures
    - at 4 ºC
    - at RT

- two corpse status
    - early postmortem status - no putrefaction
    - late postmortem status - putrefaction

- more time points: Day 0, Day 3, Day 6, Day 13

MCM
- trypan blue vital dye - 5 min at RT
- dead and living cells were manually counted using a counting chamber and a light microscope

CVA
- trypan blue vital dye included in the kit for automatic dyeing
- size limits for cell capturing were set between 8 to 20 µm
- automatic counting using the trypan blue vital dye exclusion method

- trypan blue vital dye
    - viable cells resist the dye passage through the membrane
    - dead cells are marked intensively blue

FCM
- cells were concentrated by centrifugation at 580 g for 5 min
- re-suspended in a buffer solution
- isolated cells were stained with propidium iodide
    - fluorescent dye - penetrates the impaired cytoplasmic membrane of dead or injured cells
    - binds to their nuclear DNA

- each examined tissue sample was analyzed with three parallel aliquots

Conclusions

- CVA provided the most reliable measurements and the best cost-time performance

- study confirmed the time-dependent chondrocyte viability decline
- not affected by the ambient temperature - caused by?
    - the small sample size - single donor
    - large aseptic void volume of the storage container

- Samples taken from the left auricle - microbial infection after three days of body decay at RT
    → useless in forensics - cartilaginous tissue of the head, neck, and torso

- tangential cutting with a scalpel blade inappropriate
    - blade repeatedly tends to slide over the surface or stop at wavy subchondral bone
    - cartilage shell was of an uneven shape and thickness
    - consisted predominantly of chondrocytes of the superficial and intermediate zones

→ standardized instrument to reach the whole thickness of the cartilage

- does enzymatic tissue digestion prior to the cell analysis cause additional destruction of cells?

→ comparative analysis with the cells *in situ* was necessary to evaluate possible artifacts

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TECHNICAL NOTE

PATHOLOGY/BIOLOGY

Armin Alibegović,[1] M.D., Ph.D.; Jože Balažic,[1] M.D., Ph.D.; Danijel Petrovič,[2] M.D., Ph.D.; Nevenka Kregar Velikonja,[3] Ph.D.; Rok Blagus,[4] Ph.D; Dušan Šuput,[5] M.D., Ph.D.; and Matej Drobnič,[6] M.D., Ph.D.

The Optimal Combination of Cartilage Source and Apparatus for Long-Term *In Vitro* Chondrocyte Viability Analysis

- aim - compare CVA to method that enables viability analysis on the cells *in situ*

- Live/Dead probes - confocal laser scanning microscope (CLSM)

- three donors

- knee and ankle

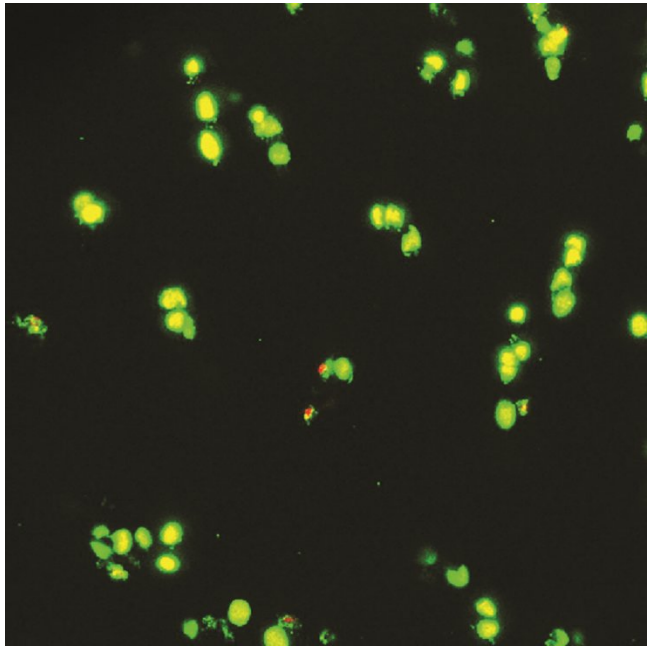
- two temperature conditions

- 1 month after the donor's death

- osteochondral cylinders - stored in a tightly closed tubes filled to the top with the cell preservation media

- approximate the conditions in dead bodies
    - limited amount of media and nutrients that were diffused to the cells
    - media was not exchanged
    - oxygen was limited and was not added
    - waste materials were not removed

CLSM



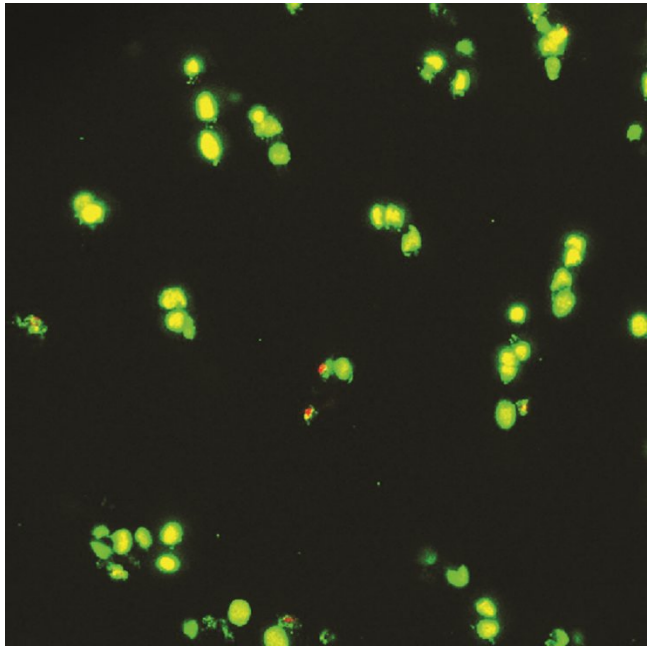
fluorogenic reagents:

- calcein-AM
  - membrane permeable nonfluorescent esterase substrate
  - passively diffuses into cytoplasm
  - after intracellular enzymatic hydrolysis remaining calcein is impermeable
  - trapped by intact cell membranes - emits a green fluorescence when excited

  → cell has an intact membrane - considered viable

- ethidium homodimer-1
  - impermeable to intact cell membranes
  - able to diffuse through the porous membranes of dying or dead cells
  - high affinity to nucleic acids and emits a bright red when excited

CLSM



- 3 of 6 stained slices were randomly selected for the analysis with CLSM

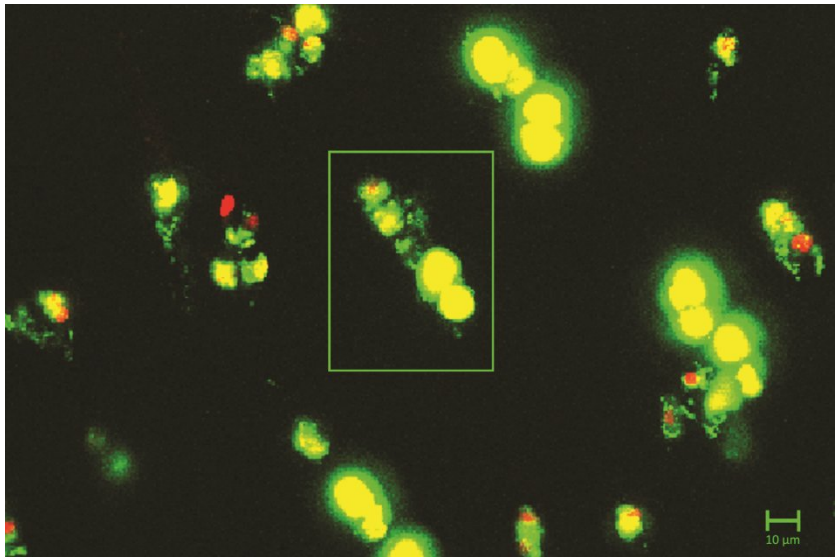
- chosen locations on each slice ~ 40 µm deep into the specimen + 400 µm away from the slice margins

- confocal micrographs with green and red colored spots, live and dead cells

- cells were counted manually

CLSM

- automatic software-based protocols for the image analysis of Live/Dead chondrocytes
- used in previous studies - faster and observer-independent analysis
- thresholding could induce a systematic error



- our study
- high magnification
- manual cell counting
- groups of green fragments without a red signal
- membrane particles of a dead cells
- fragments - could easily be mistaken for viable cells

when using automatic counting (false-positive results)

Conclusions

- cartilage from the knee joint demonstrated less variability
- knee joint was easier to access for sample procurement
- knee has a larger and thicker articular surface
- during harvesting of the ankle cartilage - coring instruments often destroyed - harder subchondral bone

	CVA	CLSM
Isolation		
Material costs		
(€)	9.6	0.7
($)	13.5	1
Time for a single sample (min)	50	16
Time for 15 samples (h)	10	4
Analysis		
Material costs		
(€)	5.6	0.1
($)	7.9	0.14
Apparatus rental price		
(€/h)	12	77.4
($/h)	16.9	109.1
Time for a single sample (min)	10	28
Time for 15 samples (h)	1	7

- CLSM in combination with Live/Dead staining provided slightly superior reliability over the CVA

- technical and cost-time issues
  - CLSM - should be reserved for basic studies
  - CVA - automatic cell counting - more user-friendly
    - high level of reliability
    - could be used in routine work

Present methods

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Viability of Human Articular Chondrocytes Harvested Postmortem: Changes with Time and Temperature of *In Vitro* Culture Conditions*

- aim – to acquire 2-month *in vitro* viability curves at different temperatures

- 16 male donors aged from 20 to 47 years
    - died of a sudden natural or violent death at the known time
        - sudden cardiac death
        - head and internal injuries in traffic or other accidents
        - accidental heroin intoxication
        - accidental or suicidal asphyxia

    - no medical history of any knee joint pathology
    - no any record of a systemic illness that could result in cartilage deterioration

- samples were procured and stored in the same way such as in presented pilot studies

- change
    - difference in antibiotics combination and dosis
    - determined after another three pilot studies
    - 5 µg/mL of vancomycin, 100 µg/mL of gentamicin, and 1 µg/mL of amphotericin B

- procured samples were randomly divided into four groups - stored at
    - 4 °C ± 2 °C
    - 11 °C ± 2 °C
    - 23 °C ± 2 °C
    - 35 °C ± 2 °C

- initial viability analyses - conducted within 36 h after the donor's death (mean 20 h)
- subsequent analyses at each temperature
    - daily - next 3 days
    - then in 3-day intervals until 63 days after the donor's death

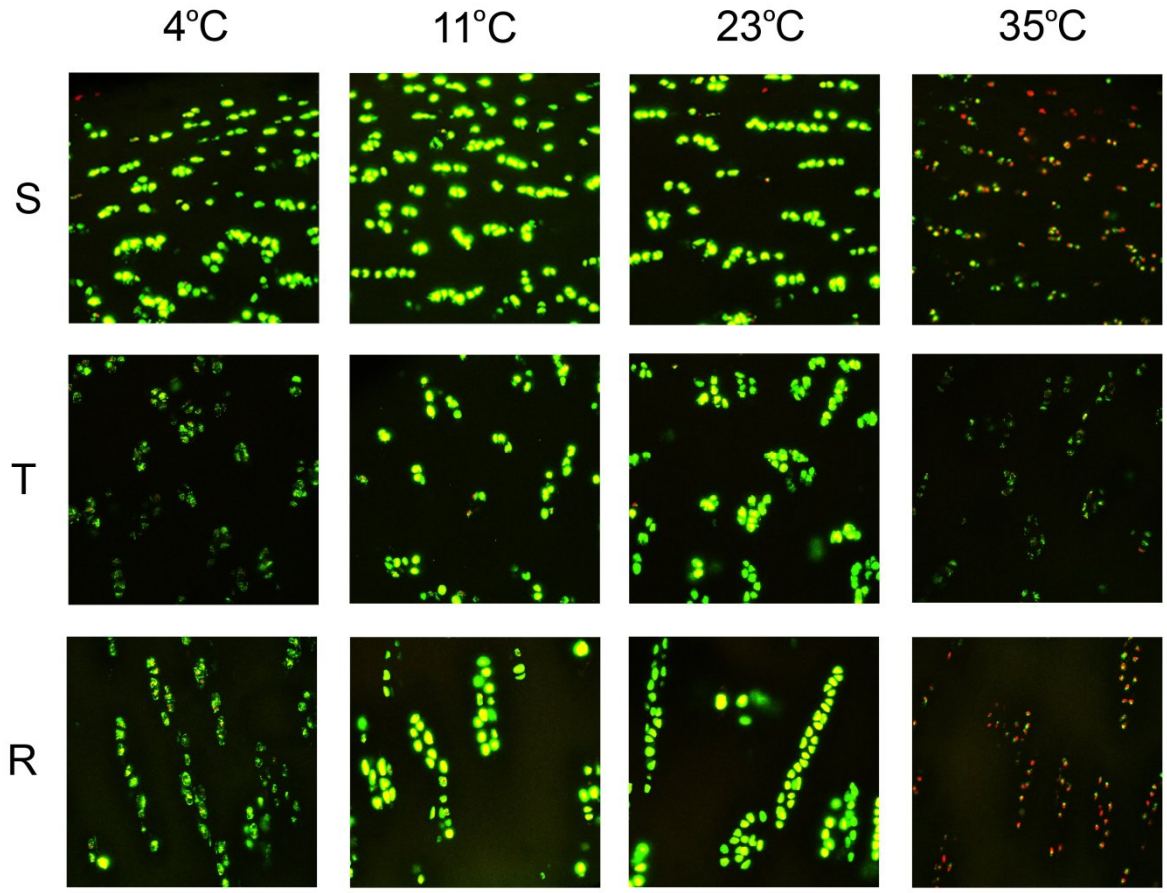
- one sample of every donor was analyzed at each time-temperature point
- obtained 1536 osteochondral cylinders - 16 donors, each with the 96 time-temperature points

- osteochondral cylinders - prepared identicaly as in previously mentioned pilot studies

- fraction of viable chondrocytes
    - calculated from three separate cell counts - one in each noncalcified cartilage zone
    - to avoid differences in cell shape, density, and metabolic activity

- viability analysis - discontinued when three consecutive samples showed no viable cell

- person unrelated to the research manually counted - viable and nonviable cells from captured micrographs



- on Day 27 after the donor's death

- 4 temperatures

- 3 articular cartilage zones (superficial, transitional, radial)

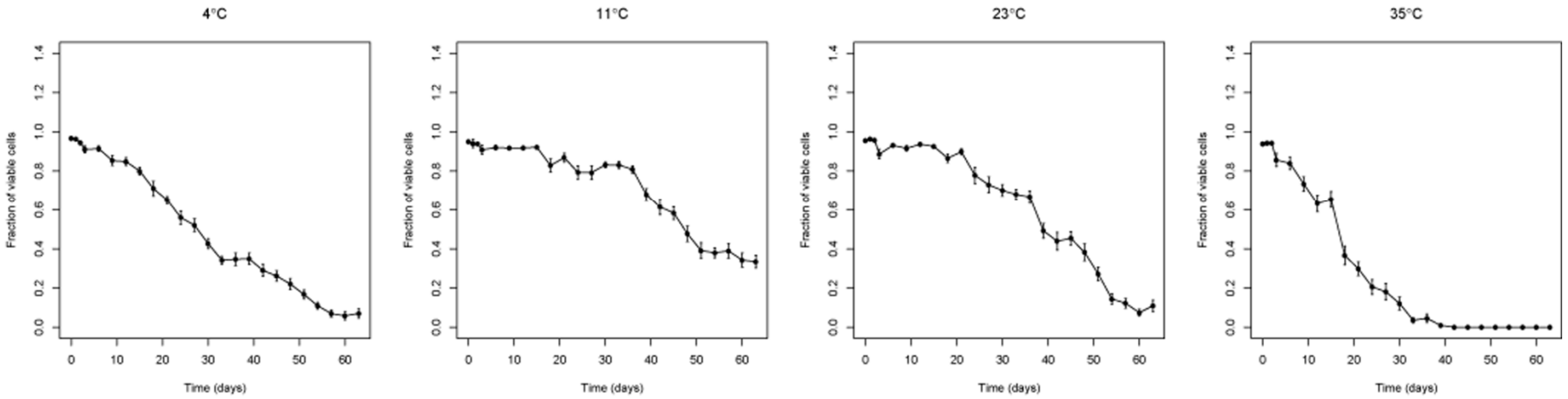
- chondrocytes in the superficial zone were the most resistant

- most of the cells in the transitional and radial zone from the sample stored at 4 °C were dead

- almost all cells from the sample stored at 35 °C were dead

- fraction of viable chondrocytes much higher in the samples stored at 11 °C and 23 °C

Results



Results

TABLE 2—*ANOVA table showing the influence of time and temperature on the knee chondrocytes viability measured using the confocal laser scanning microscope.*

	numDF	denDF	*F*-Value	*p*-Value
(Intercept)	1	1229.00	1890.25	<0.001
Time	23	1229.00	115.60	<0.001
Temperature	3	45.00	63.30	<0.001

- ANOVA - significant influence of time and temperature on the fraction of viable cells

Results

TABLE 3—*The contrasts of the cells' viability in the knee cartilage measured using the confocal laser scanning microscope at four different temperatures.*

	Estimate	Std. Error	*t*-Value	Pr(>|*t*|)
4°C vs. 11°C	0.2011	0.0298	6.7567	<0.0001
4°C vs. 23°C	0.1361	0.0299	4.5597	<0.0001
4°C vs. 35°C	−0.1873	0.0305	−6.1473	<0.0001
11°C vs. 23°C	0.0650	0.0297	2.1847	0.0342
11°C vs. 35°C	0.3884	0.0304	12.7708	<0.0001
23°C vs. 35°C	0.3234	0.0305	10.6204	<0.0001

- all pairwise comparisons between different temperatures showed significant differences

- also viability of the cells at 11 °C and 23 °C was significantly different but the magnitude of the difference was too small for practical importance

Results

TABLE 4—*The contrasts of the cells' viability in the knee cartilage measured with the confocal laser scanning microscope at different measuring time points.*

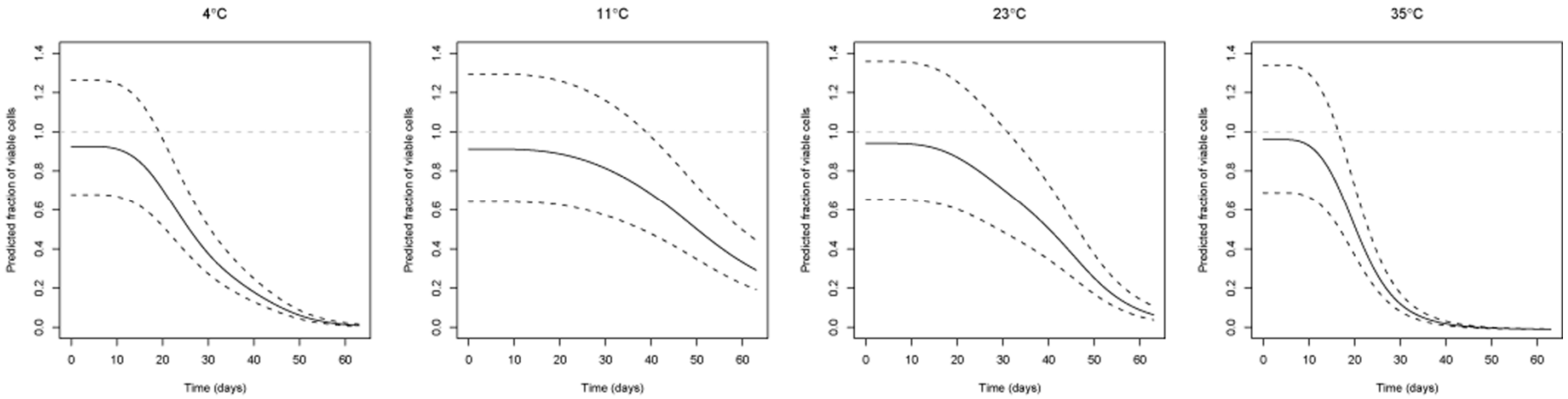
	Estimate	Std. Error	*t*-Value	Pr(>|*t*|)
Day 0 vs. Day 1	0.0053	0.0305	0.1733	0.8625
Day 0 vs. Day 2	−0.0086	0.0305	−0.2817	0.8136
Day 0 vs. Day 3	−0.0576	0.0305	−1.8883	0.0681
Day 0 vs. Day 6	−0.0513	0.0305	−1.6806	0.1020
Day 0 vs. Day 9	−0.0930	0.0305	−3.0475	0.0029
Day 0 vs. Day 12	−0.1187	0.0305	−3.8891	<0.0001
Day 0 vs. Day 15	−0.1276	0.0305	−4.1806	<0.0001
Day 0 vs. Day 18	−0.2270	0.0308	−7.3786	<0.0001
Day 0 vs. Day 21	−0.2698	0.0305	−8.8425	<0.0001
Day 0 vs. Day 24	−0.3212	0.0310	−10.3481	<0.0001
Day 0 vs. Day 27	−0.3526	0.0312	−11.3065	<0.0001
Day 0 vs. Day 30	−0.4199	0.0308	−13.6425	<0.0001
Day 0 vs. Day 33	−0.4638	0.0309	−15.0046	<0.0001
Day 0 vs. Day 36	−0.4468	0.0312	−14.3233	<0.0001
Day 0 vs. Day 39	−0.5369	0.0315	−17.0475	<0.0001
Day 0 vs. Day 42	−0.5629	0.0325	−17.3190	<0.0001
Day 0 vs. Day 45	−0.5996	0.0323	−18.5452	<0.0001
Day 0 vs. Day 48	−0.6295	0.0333	−18.9034	<0.0001
Day 0 vs. Day 51	−0.6864	0.0347	−19.7668	<0.0001
Day 0 vs. Day 54	−0.7870	0.0340	−23.1617	<0.0001
Day 0 vs. Day 57	−0.7651	0.0373	−20.5218	<0.0001
Day 0 vs. Day 60	−0.7888	0.0400	−19.7274	<0.0001
Day 0 vs. Day 63	−0.7575	0.0411	−18.4158	<0.0001

- pairwise comparisons between different time points was not significantly different for the viability of the cells from Day 0 to Day 9

- on Day 9 the viability was significantly lower than on Day 0

- same trend continued further

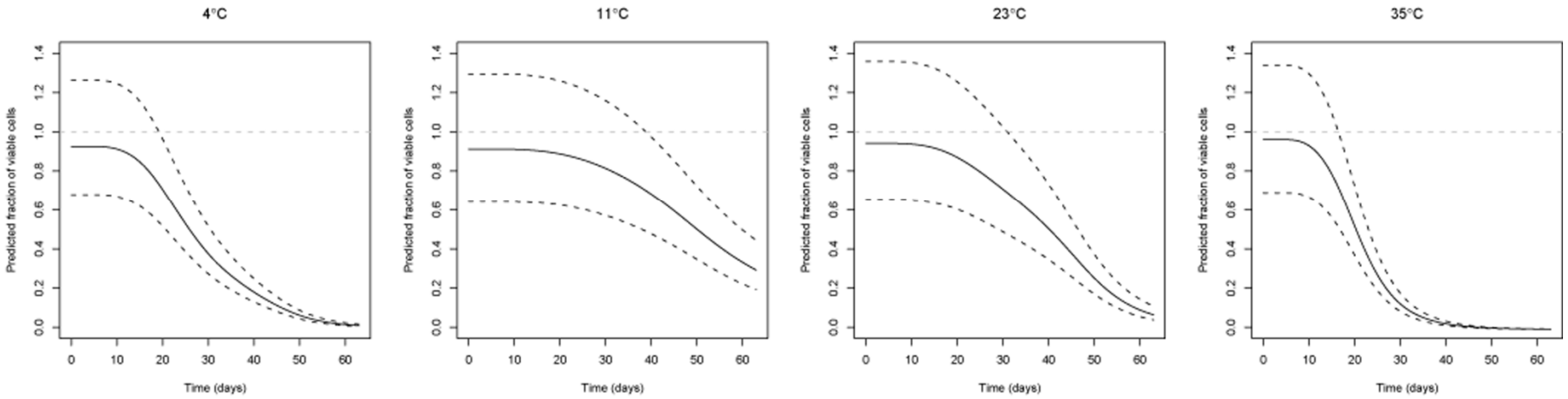
Results



- statistical calculations from the collected data were the basis for the construction of the viability curves
- according to the model that best fitted the data for each temperature

- predicted values (solid lines) + prediction intervals with 95 % accuracy (dotted lines) of chondrocytes' viability
- level of 100 % viability is marked (dotted line)

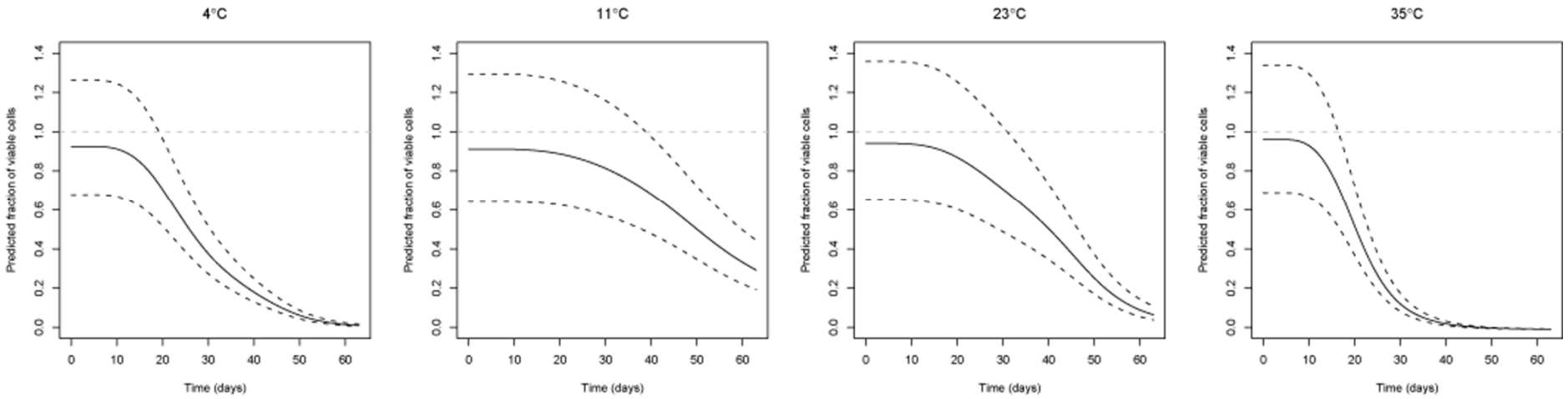
Results



- all estimated curves have a sigmoid shape

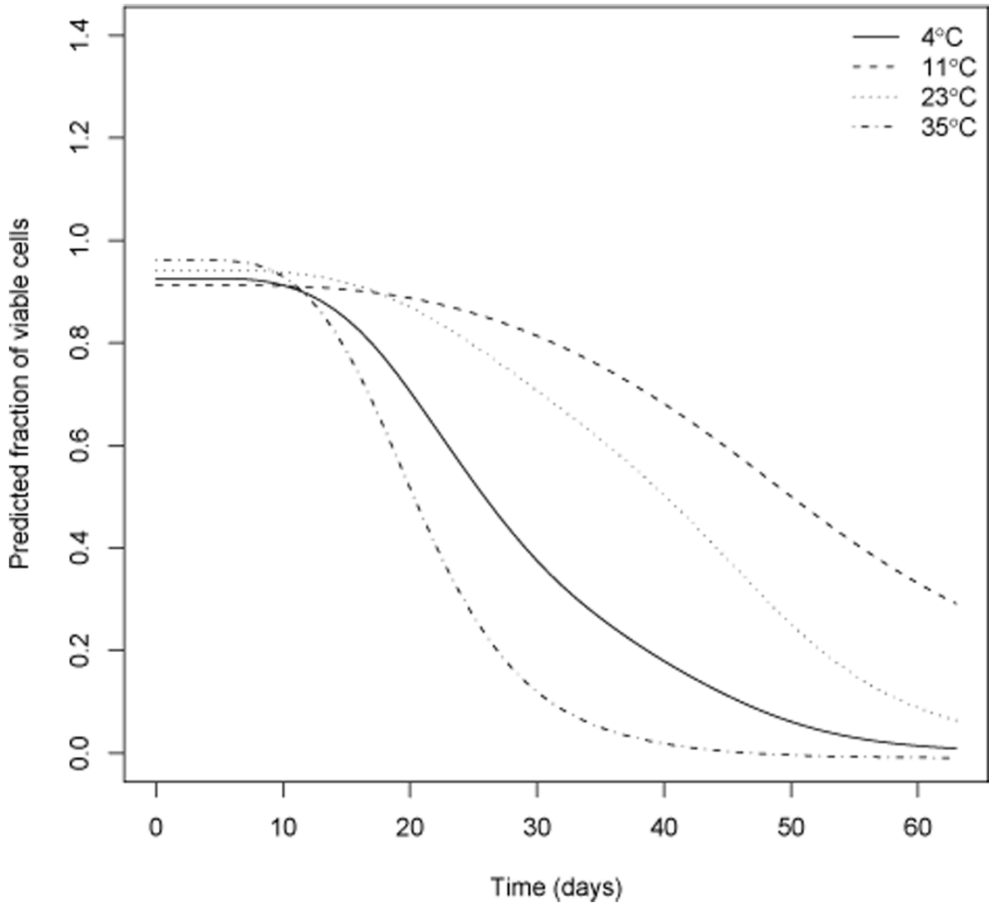
- each of them begins with a plateau - 10, 15, 12.5, and 7.5 days long at 4 °C, 11 °C, 23 °C, and 35 °C
- plateaus gradually change into a more or less linear part with different inclinations
    - steeper at 35 °C and 4 °C than at 23 °C and 11 °C

Results



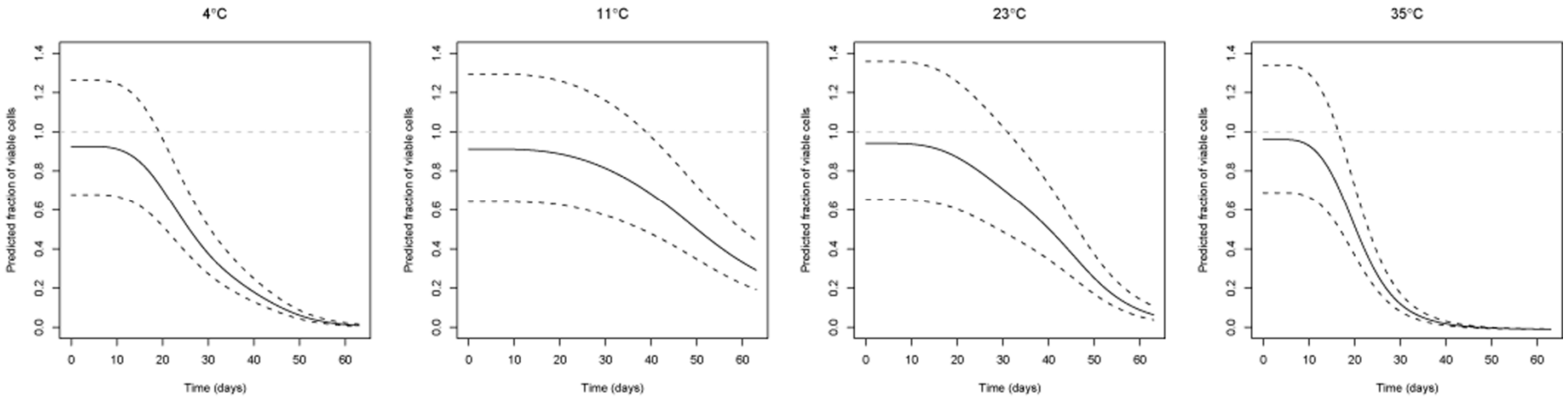
- later all curves become less steep - slowly approach null viability - especially below the level of 5 %
  - margins of the prediction intervals of survived cells below 5 % level
    - at 4 °C → 50-56 days after the first measurement
    - at 11 °C was above 30 % at the last measurement
    - at 23 °C viability curve reached the 5 % level just after Day 63
    - at 35 °C → 34-38 days after the first measurement

Results



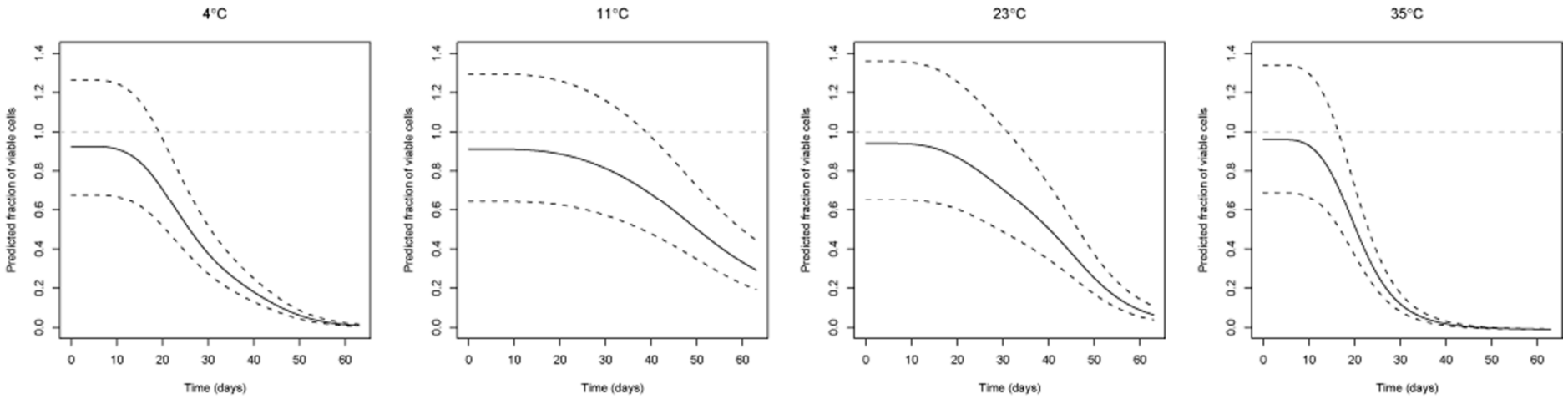
- viability curves corresponding to the ambient temperatures when plotted on the same scale

Results



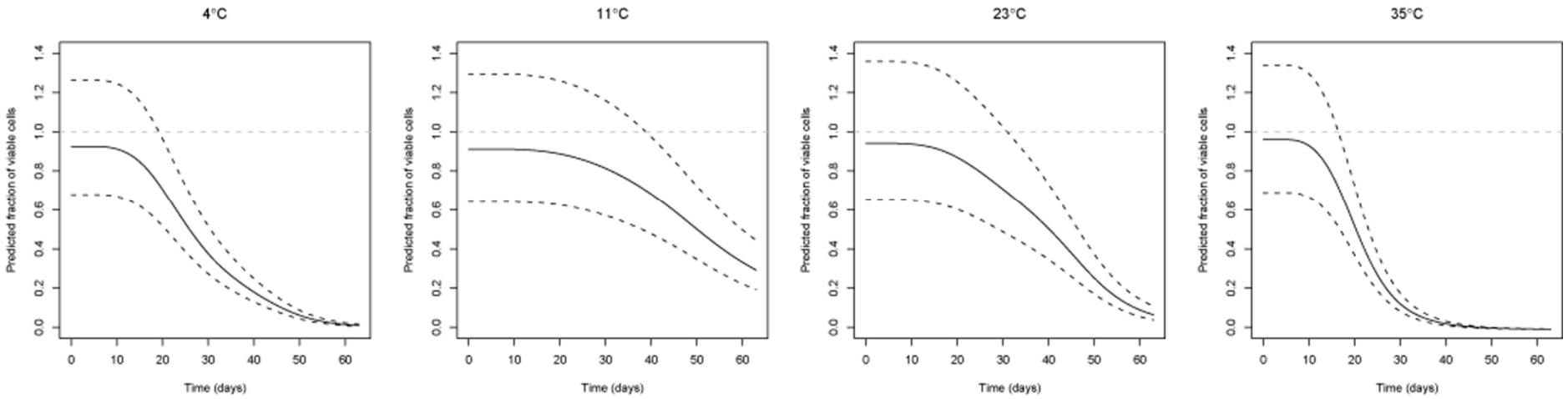
- estimated curve models could be applied to the retrograde determination of the first measurement time (theoretically representing the time of death) for the cartilage sample stored *in vitro*

Results



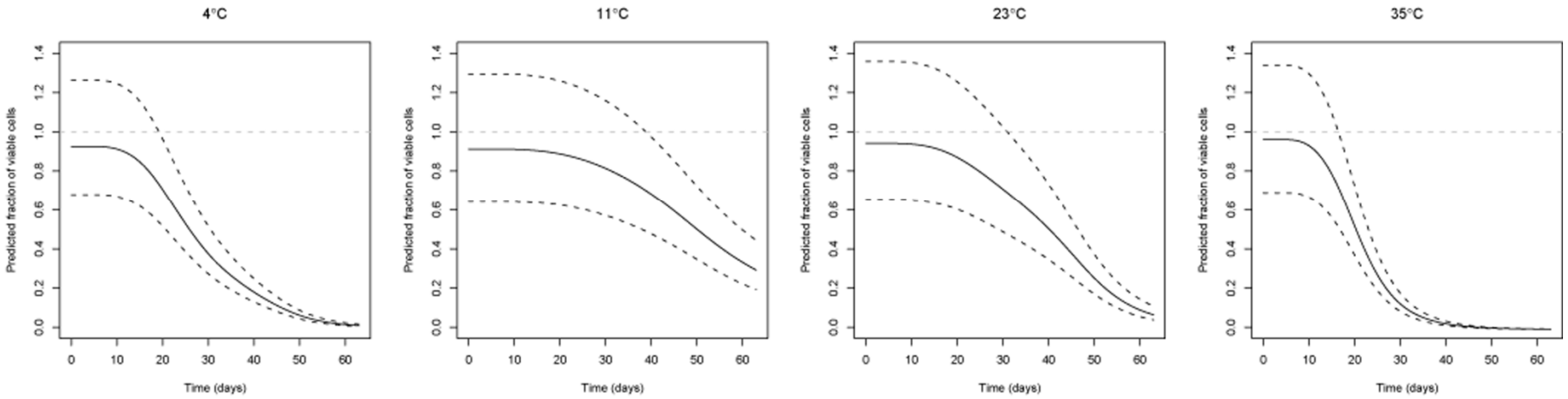
- plateau inable usage of this model - 7.5- to 15-day-long - depending on the ambient temperature

Results



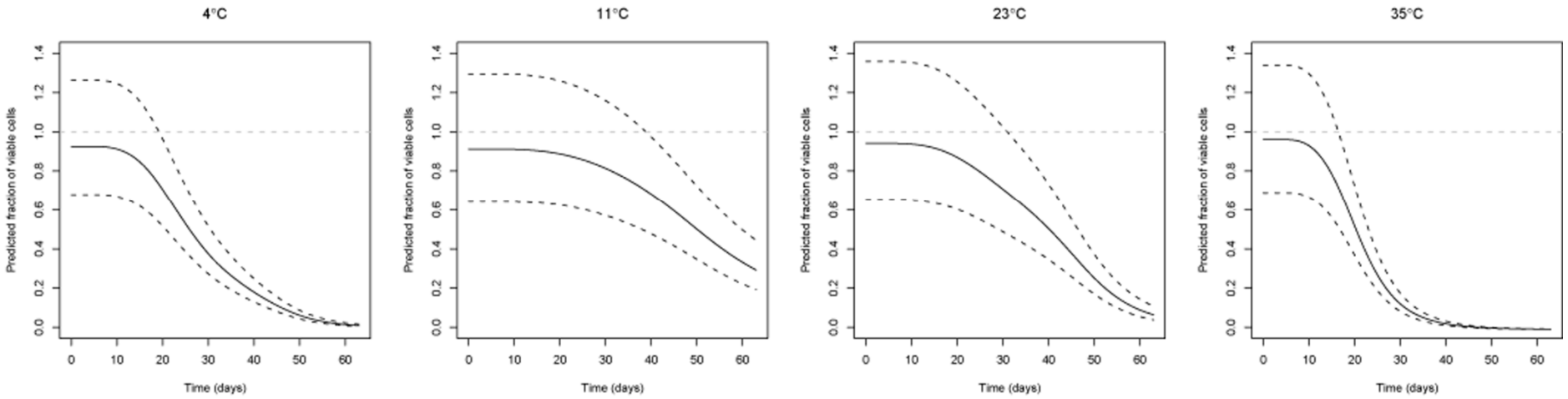
- at later time points - variability in the data is smaller - prediction intervals are narrower
- allow us to predict the time of the first measurement (time of individual death) more accurately

Results



- below the 5 % level of the surviving cells - viability curves become relatively flat
- determination of the first measurement from these parts of the curves is useless, despite a narrow prediction interval

Results



- margins of the prediction intervals for the viability curves of the samples stored at:
  - 4 °C reached the 5 % level 50-56 days after the first measurement
  - 35 °C reached the 5 % level 34-38 days after the first measurement
  - 23 °C reached the 5 % level during the last measurement
    - following the trend of the viability curve and the prediction intervals
    - estimation that the 5 % level could be reached 62-68 days after the first measurement
  - 11 °C were around 30 % at the last measurement
    - curve trend suggests
    - that 5 % level could be reached 80-90 days after the first measurement

Results

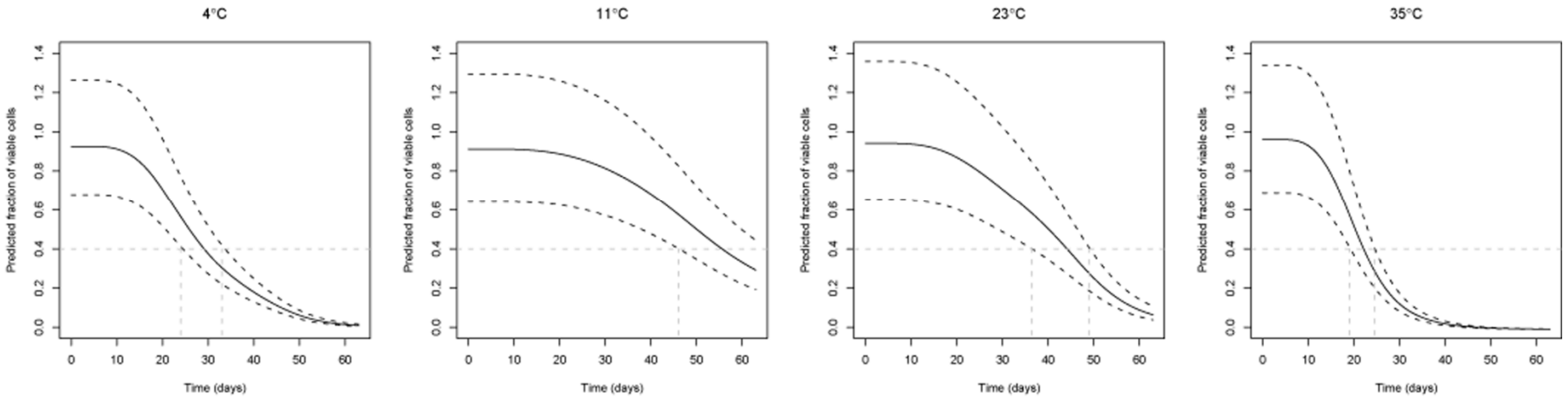
TABLE 5—*Time prediction intervals (days) at useful margin levels (fraction) of the chondrocytes' viability curves at four different temperatures.*

	Upper Limit		Lower Limit	
Temperature	Fraction	Day	Fraction	Day
4°C	0.67	10–26	0.05	50–56*
11°C	0.64	15–53	0.05	80–90†
23°C	0.65	12.5–42.5	0.05	62–68†
35°C	0.69	7.5–20.5	0.05	34–38*

*Values set from the graphs.
†Values estimated on the basis of the curves' trend.

- presented model is useful after the proportion of surviving cells falls below 64%-69% (upper limit), depending on the ambient temperature, to the level of 5 % (lower limit)

Results



- practical usage of this model could present next example

- measured 40 % chondrocyte viability showed prediction intervals with the 95 % accuracy that the first measurement, representing the time of the donor's death, from the samples stored at:
    - 4 ºC was before 29±5 days
    - 11 ºC was before 56±10 days
    - 23 ºC was before 44±7 days
    - 35 ºC was before 22±3 days

Conclusions

- obtained results confirmed that the chondrocytes' viability in the cartilage of a human corpse might be used for the determination of the time since death as it decreases constantly with time and under the influence of the ambient temperature

- main weakness is the *in vitro* setup of the study, but it was necessary
    - to put the chondrocytes from different samples in the same conditions
    - to exclude the influence of the internal and external, ante-mortem and postmortem unpredictable factors
        - such as different types and amounts of bacteria during body decomposition

- presented *in vitro* estimated curves serve only as foundations for the coming *in corpore* study which
    - should be extensive
    - would include, beside ambient temperature, also the other factors in the natural environment

- in the manuscript were discussed numerous other problems and statements

Future methods

- cartilage could be determined as a compartment
- cartilage could be used for the determination of the PMI

- chondrocytes' viability decreases gradually and steadily as a function of time and temperature
- objective determination of the time of an individual's death several weeks after the moment of dying

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However, broader study of cartilage as a possible new parameter for PMI determination in forensic medicine has not been performed.

Since the corpse is exposed to a wide variety of environmental conditions that cannot be controlled, it was necessary to perform a study in supervised *in vitro* conditions before an extensive *in corpore* study.

Past, present and future methods for assessment of chondrocytes' viability (a new parameter for determination of the postmortem interval?)

Armin Alibegović

Institute of forensic medicine

University *of Ljubljana*
Faculty *of Medicine*