INTRODUCTION:
Articular cartilage damage may start in young adults as a result of abnormal joint loading. Early damage may progress into severe osteoarthritis if poor mechanical conditions persist. Unfortunately, our knowledge on the relationship between mechanical loading, the early onset of cartilage damage and the progression thereof is limited. We previously showed that indentation initiates collagen damage in cartilage [1]. In this study, we aim to identify threshold levels for the initiation of cartilage damage and its progression by monitoring tissue softening as a result of various levels of impact loading.

METHODS:
Osteochondral plugs (ø7.5 mm, 6 mm high) were obtained from young bovine carpometacarpal joints and stored at -20°C until testing. After thawing to room temperature, samples were left to equilibrate in PBS. After cartilage thickness was assessed using stereomicroscopy, samples were fixed using dental cement to the bottom of a 12 mm high bath filled with PBS. Samples were indented with a stainless steel spherical indenter (tip radius, 1 mm), using a tensile testing machine (ElectroForce LM1 TestBench, Bose Corporation, Minnesota, USA).

The experiment contained one control group in which no damage was induced and four experimental groups in which different loading regimes were applied. All groups received a baseline loading of 5% indentation. In each 1500 s, a 600 s period of 10% indentation was applied, during which relaxation was monitored, followed by 900 s period of baseline indentation. After 600 s during this phase, the indentor was lifted from the sample to obtain a reference 0 N force measurement. These cycles were repeated 11 times (Fig. 1a, blue line). Superimposed on this control-loading regime (n = 6), the four experimental groups received impact loading during each baseline period. This was either a constant load of 3 N (n = 5), 6 N (n = 5) or 15 N (n = 6), or an increasing load (n = 7) from 2 to 13 N in 11 steps (Fig. 1a, red lines).

To assess the mechanical properties of the cartilage, peak and relaxation forces were monitored during each 10% indentation period (Fig. 1b). Forces were normalized to the first cycle value and expressed as a function of loading cycle. After each experiment, the tissue was evaluated for GAG and collagen contents biochemically. Histology was performed to assess visible damage to the samples, and col2-3/4m and col2-Ch short immunostainings were performed to monitor possible damage to the collagen network [1].

RESULTS:
The 29 samples were on average 0.95 mm thick and there was no significant difference in water, collagen and GAG content between the different group samples. In the control group, peak and equilibrium forces did not change with the loading cycle, indicating that no tissue softening occurred as a consequence of the 10% indentation period (Fig. 2). The threshold indentation loading for initiation of tissue softening in this experiment is around 6 N; some samples were more susceptible to softening than others: four samples showed softening, one did not. This explains the slightly higher standard deviation in this group. Indeed, all samples that received 15 N or the increasing impact load showed considerable decreases in peak and equilibrium forces during the 10% indentation loading that followed impact loading. Most of the loss in peak and equilibrium forces occurred during the first few loading cycles, and softening seemed to slowly progress when the same loading was repeatedly applied. The group that received increasing impact showed that softening increases with the applied force. (Imuno)histology did not reveal signs of visible collagen damage to any of the samples.

DISCUSSION:
We designed an experimental protocol to monitor softening in cartilage in response to excessive indentation loading. Using this protocol, we showed that a particular loading threshold exists above which tissue softening starts to occur in articular cartilage. Above this threshold, both the peak and equilibrium values of the reaction force decrease concurrently. Although this may indicate that damage is occurring in the tissue, histological and immunohistochemical evaluations did not reveal visible signs of collagen fiber breakage in the tissue. With a comparable loading protocol and using higher forces, we previously detected early collagen damage in the cartilage [1]. This indicates that softening is a very early effect of tissue overloading in articular cartilage, that becomes apparent even before structural changes can be observed (immuno)histologically.

SIGNIFICANCE:
Cartilage softening may be one of the earliest signs of articular cartilage damage. Therefore, identifying thresholds for the onset of softening, monitoring the progression of softening with ongoing tissue loading, and ultimately linking softening to changes and/or damage in the constituents of cartilage is an important step towards understanding the etiology of cartilage damage and osteoarthritis.

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REFERENCES: