Changes in tumor stiffness for early prediction of tumor response to sorafenib: a proof-of-concept study with elastosonography in an animal model of Hepatocellular Carcinoma (HCC).

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**ABSTRACT**

**Background and aims:** Sorafenib is the reference therapy for advanced Hepatocellular Carcinoma (HCC). No method exists to predict in the very early period subsequent individual response. Starting from the clinical experience in humans that subcutaneous metastases may rapidly change consistency under sorafenib and that elastosonography a new ultrasound based technique allows assessment of tissue stiffness, we investigated the role of elastosonography in the very early prediction of tumor response to sorafenib in a HCC animal model.

**Methods:** HCC (Huh7 cells) subcutaneous xenografting in mice was utilized. Mice were randomized to vehicle or treatment with sorafenib when tumor size was 5-10 mm. Elastosonography (Mylab 70XVG, Esaote, Genova, Italy) of the whole tumor mass on a sagittal plane with a 10 MHz linear transducer was performed at different time points from treatment start (day 0, +2, +4, +7 and +14) until mice were sacrificed (day +14), with the operator blind to treatment. In order to overcome variability in absolute elasticity measurement when assessing changes over time, values were expressed in arbitrary units as relative stiffness of the tumor tissue in comparison to the stiffness of a standard reference stand-off pad lying on the skin over the tumor.

**Results:** Sor-treated mice showed a smaller tumor size increase at day +14 in comparison to vehicle-treated (tumor volume increase +192.76% vs +747.56%, p=0.06). Among Sor-treated tumors, 6 mice showed a better response to treatment than the other 4 (increase in volume +177% vs +553%, p=0.011). At day +2, median tumor elasticity increased in Sor-treated group (+6.69%, range –30.17–+58.51%), while decreased in the vehicle group (-3.19%, range –53.32–+37.94%) leading to a significant difference in absolute values (p=0.034). From this time point onward, elasticity decreased in both groups, with similar speed over time, not being statistically different anymore. In Sor-treated mice all 6 best responders at day 14 showed an increase in elasticity at day +2 (ranging from +3.30% to +58.51%) in comparison to baseline, whereas 3 of
the 4 poorer responders showed a decrease. Interestingly, these 3 tumours showed elasticity values higher than responder tumours at day 0.

**Conclusions:** Elastosonography appears a promising non-invasive new technique for the early prediction of HCC tumor response to sorafenib. Indeed, we proved that responder tumours are characterized by an early increase in elasticity. The possibility to distinguish a priori between responders and non responders based on the higher elasticity of the latter needs to be validated in ad-hoc experiments as well as a confirmation of our results in humans is warranted.
INTRODUCTION

HepatoCellular Carcinoma (HCC) is one of the most common solid cancer worldwide, being the third cancer-related mortality cause.\(^1,2\) Its incidence is increasing in Western countries mainly due to the high prevalence of Hepatitis C Virus (HCV) infection. Potential curative therapies are currently offered to patients with very early/early stages HCC, according to the BCLC (Barcelona-Clinic Liver Cancer) staging system, which represent only one third of newly diagnosed HCC patients.\(^3\) Only supportive care were offered to the remaining patients until 2007, when the clinical management of HCC radically changed thanks to the SHARP (Sorafenib HCC Assessment Randomized Protocol) trial that showed a survival benefit in patients with advanced HCC treated with sorafenib versus placebo.\(^2\) Sorafenib is a multikinase inhibitor of Vascular Endotelial Growth Factor Receptor 2 (VEGFR 2), Platelet Derived Growth Factor Receptor (PDGFR), Raf-1, B-Raf and c-kit among others,\(^4\) currently adopted as standard care in patients with advanced HCC.\(^5\)

Antiangiogenic treatments, such as sorafenib, are addressed to block signals related to neoangiogenesis rather than to provide a direct cell killing effect. Since that they can be effective even in absence of tumor shrinkage, WHO (World Health Organization) and RECIST criteria cannot be used in this setting.\(^6,7\) These criteria, which are based on dimensional evaluation following therapy, were amended in 2000 by a panel of experts on HCC during the European Association for the Study of the Liver (EASL) conference. The new suggested concept aimed to take into account the intratumoral necrosis induced by the treatment, rather than the reduction in tumour size. Response to treatment was then evaluated measuring the reduction in viable tumor area, assessed using contrast-enhanced radiological techniques. A modified version of RECIST criteria was created in 2008 under the auspices of the American Association for the Study of Liver Diseasase (AASLD) (m-RECIST) in order to provide a common approach for designing clinical trials and to standardize imaging techniques for response assessment.\(^8\) Even if promising results are available concerning contrast-enhanced ultrasonography and its quantification,\(^9,10\) up
to now only “heavy” radiological techniques (contrast-enhanced computed tomography and magnetic resonance) are commonly accepted in this setting.

Elastosonography is an ultrasound-based technique able to evaluate in vivo the elastic proprieties of a tissue measuring the strain in response to an external compression. This method has been successfully employed in superficial tissues, such as thyroid or breast, for the differentiation between benign and malignant nodules\textsuperscript{11-14} whilst its role the diagnosis of HCC\textsuperscript{15,16} and in the evaluation of hepatic fibrosis remains controversial\textsuperscript{17,18}.

A softening of a tumoral mass during therapy is often the first objective evidence of tumour response\textsuperscript{19,23}. So far, only subjective sensings are described and no data are available concerning an objective quantification of changes in tumour consistence. Thus, the present study aims to evaluate whether elastosonography may be a useful tool in the response assessment to antiangiogenic treatment in an experimental model of HCC.
MATERIALS AND METHODS

Experimental model

Human cell line Huh7, kindly provided by Dr. Porretti, was maintained and expanded using standard cell culture technique in high glucose Dulbecco’s Modified Eagle Medium supplemented with L-glutamine, 1% ampicillin/amphotericin B and 10% fetal bovine serum (Gibco, Italy). The model was established by subcutaneous injection of $5 \times 10^6$ cells suspended in sterile phosphate-buffered saline (Gibco, Italy) for a total volume of 0.2 mL per injection into the right flank of 6-8 weeks old female mice (Charles River, Calco, Italy). During the experiments, the mice were maintained with regular mouse chow and water ad libitum in a temperature-controlled room under a 12-hour light/dark circle and specific pathogen-free circumstances. Mice were randomized to vehicle or treatment with sorafenib (BAY 43-9006; Bayer, Germany) at a dosage of 62 mg/Kg by oral gavage daily. Sorafenib was formulated as previously described. Growth of established xenografts was monitored at least twice weekly by ultrasound. Mice were divided into 2 groups: in group A, treatment started when tumor reached 5-10 mm in diameter and lasted for 14 days whereas, in group B, treatment started when tumor reached 12-18 mm in diameter and lasted for 2 days. The internal animal welfare committee approved the experimental protocol.

Ultrasound and elastosonography imaging experiments

Ultrasound examinations were performed using a MyLab70 XVG (Esaote, Genova, Italy) equipped with a 4-13 MHz probe. Mice were anesthetized intraperitoneally with 0.2 mL of a solution constituted by one part of ketamine 10% (Ketavet, Intervent Production s.r.l.), one part of xylazine 20 mg/mL (Rompun, Bayer) and eight parts of sterile water. The anesthetized animals were placed on a heating support in order to keep constant the temperature for all the duration of the experiment. For B-mode examinations, the tumours were covered with ultrasound
gel. Volume was calculated using the formula: height x width x thickness/2 measured by ultrasound, considering the respective biggest diameter.

Elastosonography was performed by a single operator by selecting “ElaXto Ratio” modality on the ultrasound system screen. This technique allows the differentiation among tissues, representing different deformations subsequent to a manual strain applied perpendicularly by the operator as colour-coded images. The same condition of brightness, contrast, intensity and gain were used in all the examinations. For elastosonography, the probe was placed on a pad by interposing a thin quantity of ultrasound gel between the probe and the pad and between the pad and the tumour, paying attention in scanning the biggest section of the tumour. The pad was used to correlate the elasticity of the tumor with a reference elasticity. Results were considered reliable when the warning light spring on the screen turned from grey to green, which meant an adequate compression level. The equipment displays two images simultaneously: the conventional B-mode image and an image where the Region Of Interest (ROI) appears in a chromatic scale that identifies different levels of elasticity. The software requires to identify two ROIs (drawn by a second operator), one including the almost totality of the pad surface (ELX1) and the other surrounding the entire tumour surface (ELX2) (Fig.1).
Figure 1. ElaXto modality. Two ROIs have to be drawn on the colour-coded image by the operator. ELX2/1 value (the ratio between the ROI of the tumour and that of the pad) is provided in real-time as well as the histogram representing the distribution of the colours in each ROI.

Results are provided in real time by dividing ELX2 for ELX1. Resulting values are directly proportional to the elasticity of the tumor compared to the pad. Also the histogram of the distributions of the colours inside the ROIs is provided. Three valid measurements were performed in each tumor and the mean value was used for following analyses. All the procedure lasted approximately 2 minutes per mice. The same procedure protocol was repeated at different time points starting from the beginning of the treatment (0, +2, +4, +7 and +14 days in group A and 0 and +2 in group B).
Necropsy

At day +14 for group A and at day +2 for group B after the last measurement and still under anaesthesia, animals were euthanized by 0.1 mL of a solution of embutramide, mebezonium iodide and tetracaine hydrochloride (Tanax, Intervet Italia s.r.l.). A slice of all group A tumours were frozen in liquid nitrogen vapour and stored at –80°C.

Western-Blot

Two monoclonal antibodies (55B11 and 20G11, Cell Signaling Technology, Inc. Danversa, MA, USA) against VEGFR2 (diluted at 1:1000) and phospho-p44/42 MAPK (Erk1/2; Thr202/Tyr204) (diluted at 1:1000) were incubated separately for 16 hours at 4°C. A horseradish conjugated secondary antibody (labeled polymer-HRP antimouse, Envision system DAKO Cytomation, Carpinteria, CA, USA) was incubated for 45 minutes at room temperature and the corresponding band was revealed using the enhanced chemoluminescence method (Amersham, UK). Digital images of autoradiographies were acquired with Fluor-S MultiImager (Bio-Rad, Hercules, CA, USA) and band signals were acquired in the linear range of the scanner using a specific densitometric software (Quantity-one, Bio-Rad, Hercules, CA, USA). Images were calibrated against a reference autoradiography and given in relative density units. After autoradiography acquisition, the membranes were stripped and reprobed for two hours at room temperature with anti b-actin antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) to normalize protein loading. A ratio between VEGFR2 or phospho-ERK and b-actin corresponding bands was used to quantify the levels of each protein (normalized value). This ratio was divided by b-actin levels of HuH7 line in each blot in order to compare the results in different running blot (absolute value).
Statistical evaluation

Data are presented as median and min-max in round brackets. Differences between groups were compared using the Mann-Whitney test (2-tailed). Differences among different time points in the same group were compared using the Wilcoxon signed Rank test. Percentage delta was calculated using the formula \[\frac{(\text{final value}-\text{starting value})}{\text{starting value}}\]%\%. P<0.05 was considered significant. Statistical analysis was performed using SPSS 16.0 (Chicago IL, USA).
RESULTS

Group A.

Group A comprised 19 mice, 10 in the treatment group and 9 in the vehicle group. Tumor volume at day 0 was 151.09 mmc (66.83-602.58). At day +14, tumor volume was 608 mmc (333.3-1799.88) in treatment group and 893.43 mmc (218.67-1996.70) in vehicle group with a growth percentage delta of 192.76% (84.72-739.48) in treatment group and 747.56% (73.47-2887.62) in vehicle group.

Elasticity tended to increase in the treatment group at day +2 compared to day 0 while a reduction was evident in the vehicle group (Table 1). ELX2/1 values at day +2 were statistically different between the two groups ($p=0.034$).

<table>
<thead>
<tr>
<th>ELX2/1</th>
<th>Treatment group</th>
<th>Vehicle group</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 0</td>
<td>1.31 (1.07-1.97)</td>
<td>1.25 (1.07-1.81)</td>
<td>n.s.</td>
</tr>
<tr>
<td>day +2</td>
<td>1.38 (1.15-1.99)</td>
<td>1.14 (0.75-1.56)</td>
<td>0.034</td>
</tr>
<tr>
<td>day +4</td>
<td>1.31 (1.12-1.87)</td>
<td>1.16 (1.03-1.44)</td>
<td>n.s.</td>
</tr>
<tr>
<td>day +7</td>
<td>1.25 (1.08-1.79)</td>
<td>1.19 (0.94-1.59)</td>
<td>n.s.</td>
</tr>
<tr>
<td>day +14</td>
<td>1.16 (0.70-1.74)</td>
<td>1.09 (0.75-1.35)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Table 1. ELX2/1 values in the treatment and in the vehicle group. A difference between the two groups was evident only at day +2 ($p=0.034$).

In treatment group, after an early increase from day 0 to day +2, elasticity decreased from day +2 to day +14 (Fig.2). Indeed, ELX2/1 values at day +14 were statistically different compared to day 0 ($p=0.028$).

In vehicle group, after an early decrease from day 0 to day +2, then elasticity remained quite constant over time without statistical difference between day 0 and day +14 ELX2/1 values.
Fig. 2. ELX2/1 trend over time in the treatment group (green line) and in the vehicle group (blue line). A statistical difference was evident at day +2 between the two groups ($p=0.034$), showing a higher elasticity of tumours in the treatment group with respect to the vehicle group. ELX2/1 tended to decrease in the treatment group comparing day 0 and day +14 ($p=0.028$).

A sub-analysis was performed in treatment group dividing tumours with respect to growth percentage delta. If the growth percentage delta was <200%, tumours were considered as responder (6 cases, ranging from 85% to 199%) whilst non responder if the growth percentage delta was >350% (4 cases, ranging from 382% to 739%).

Volume at day +14 was 523.56 (333.33-1799.88) in the responder group and 761.69 (474.30-1154.16) in the non responder group. ELX2/1 was 1.31 (1.07-1.97) at day 0. All responder tumours showed an increase in ELX2/1 from day 0 to day +2. Among non responder tumours, 3 out of 4 showed at D0 higher values than responder tumours. Furthermore, a decrease of ELX2/1 was evident in these 3 cases whilst an increase was seen in the remaining one (Table 2 and Fig.3).
<table>
<thead>
<tr>
<th></th>
<th>ELX2/1 at D0</th>
<th>ELX2/1 at D2</th>
<th>ELX2/1 %Delta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responder (R) tumours</td>
<td>1.36</td>
<td>1.54</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>1.99</td>
<td>58.5</td>
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<td></td>
<td>1.27</td>
<td>1.38</td>
<td>8.9</td>
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<td>1.27</td>
<td>1.33</td>
<td>4.5</td>
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<td></td>
<td>1.35</td>
<td>1.52</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td>1.11</td>
<td>1.15</td>
<td>3.3</td>
</tr>
<tr>
<td>Non Responder (NR) tumours</td>
<td>1.97</td>
<td>1.37</td>
<td>-30.2</td>
</tr>
<tr>
<td></td>
<td>1.81</td>
<td>1.47</td>
<td>-18.9</td>
</tr>
<tr>
<td></td>
<td>1.60</td>
<td>1.23</td>
<td>-22.8</td>
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<tr>
<td></td>
<td>1.07</td>
<td>1.23</td>
<td>15.3</td>
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<tr>
<td>Median values R tumours</td>
<td>1.27</td>
<td>1.45</td>
<td>10.9</td>
</tr>
<tr>
<td>Median values NR tumours</td>
<td>1.71</td>
<td>1.30</td>
<td>-20.8</td>
</tr>
</tbody>
</table>

Table 2. Individual and median values of ELX2/1 at day 0 and day +2. Percentage delta of ELX2/1 was also calculated. Three out of 4 non responder (NR) tumours showed at day 0 higher values than responder (R) tumours, with a decrease at day +2. On the contrary, all R tumours showed an increase in ELX2/1 values from day 0 to day +2.
Fig.3. Relationship between growth percentage delta at day +14 and percentage delta of ELX2/1 at day +2 in the treatment group, comparing responder (blue circles) and non responder tumours (green circles). A decrease in ELX2/1 values at day +2 with respect to day 0 was evident in 3 out of 4 non responder tumours whilst all responder tumours showed an increase in ELX2/1 values.

At day +14, responder tumours showed ELX2/1 values comparable to the vehicle group (1.04, 0.70-1.32 and 1.09, 0.75-1.35) whilst non responder tumours showed higher values (1.46, 1.10-1.74).
Western-Blot

Western blot analysis for VEGFR2 and phospho-ERK were performed for 16 tumor slices (and for the cell line) at necropsy. Two mice of the treated group and one of the vehicle group were not included in the western-blot analysis because of the impossibility to analyze more than 9 bands at the same time. All the results are listed in Table 3. Concerning both VEGFR2 and both phospho-ERK, no statistical difference was found between the treatment and the vehicle group and between responder and non responder group. Nevertheless, non responder group showed higher values of VEGFR2 and phospho-ERK than both responder and both vehicle group.

<table>
<thead>
<tr>
<th></th>
<th>Treated group</th>
<th>Vehicle group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non responders</td>
<td>Responders</td>
</tr>
<tr>
<td>Normalized VEGFR2</td>
<td>4.269 (0.616-26.063)</td>
<td>1.043 (0.134-1.463)</td>
</tr>
<tr>
<td>Absolute VEGFR2</td>
<td>0.200 (0.029-1.218)</td>
<td>0.049 (0.006-0.068)</td>
</tr>
<tr>
<td>Normalized phospho-ERK</td>
<td>5.346 (0.570-10.646)</td>
<td>0.944 (0.467-1.216)</td>
</tr>
<tr>
<td>Absolute phospho-ERK</td>
<td>0.250 (0.027-0.498)</td>
<td>0.044 (0.022-0.057)</td>
</tr>
</tbody>
</table>

Table 3. Results of quantification of western blot analysis. Median and min-max (round brackets) values are expressed as arbitrary units. No statistical difference was found between western blot values among the three groups (non-responder, responder and vehicle groups). However, even in absence of statistical significance, probably related to the small number of mice within each group, higher values of VEGFR2 and phospho-ERK were seen in non responders group, compared with responder and vehicle group.
**Group B**

Group B comprised 8 mice, 4 in the treatment group and 4 in the vehicle group. Tumour volume at day 0 was 1078.40 mmc (608.75-1795.07). At day +2, tumour volume was 1365.00 mmc (1118.29-1995.15) in the treatment group and 1134.43 (1010.63-1782.15) in the vehicle group with a growth percentage delta of 15.03% (-1.73-36.37) in the treatment group and 16.74% (0.72-66.02) in the vehicle group.

ELX2/1 was 1.16 (0.90-1.48) at day 0. At day +2, ELX2/1 in the treatment group was 1.37 (1.06-1.58) with a percentage delta of 17.76% (-20.45-76.21) whereas 1.20 (0.94-1.28) in the vehicle group with a percentage delta of –3.81% (-23.20-15.90) (Fig.4). No statistical difference was achieved between ELX2/1 values of the two groups nor at day 0 neither at day +2. Nevertheless, the same trend that emerged in Group A tumours was present also in Group B tumours, that is the increase in ELX2/1 values from day 0 to day +2 in the treatment group and the decrease in the vehicle group.

![Figure 4. Percentage delta of ELX2/1 comparing the treatment group and the vehicle group in Group B.](image)
DISCUSSION

Our study demonstrated that elastosonography appears as a promising tool in the early prediction of response to antiangiogenic treatment. The possibility to quantify the elastic proprieties of a mass allowed to improve the non invasive diagnosis of thyroid and breast nodules due to a higher stiffness of malignant masses with respect to benign ones but controversial results emerged concerning the liver. Possible limitations for its use in hepatic imaging may be related to the high frequency probes on which elastosonography is available. Whist the elasticity of deep lesions still remains impossible to be evaluated, the software here used allows to analyze lesions located until 10 cm in depth. Thus, the majority of focal liver lesions may be studied through an accurate selection of the scan approach. The second limitation is related to the software itself, which implies the comparative analysis between the lesion and the surrounding parenchyma. The aim of the software is not only to measure the compression induced in the tissue, which involves uniquely a change in dimension, but rather the deformation, which involves a change in shape. The elastosonographic images are created on the basis of modifications of radiofrequency signals during the compression and the release of the probe, by comparing the deformation of a structure with the surrounding tissue included in a ROI. Thus, the analysis is relative and not absolute. Liver parenchyma may be affected by several conditions which result in change of stiffness, mainly related to fibrosis deposition. Conditions other than fibrosis may affect reliability of the results, too. Among these, some authors reported the influence of cholestasis or right heart failure on Transient Elastography results but no data are actually available concerning elastosonography. HCC arises in the majority of cases on cirrhosis, a condition in which Fibroscan values range from 12-14 kPa to 75 kPa (the higher value provided by the device), reflecting the dynamic fibrosis process. For these reasons, absolute elastosonography values of focal liver lesions derived from different patients are not comparable at present. Nevertheless, longitudinal evaluation of a lesion can be performed, whichever the baseline value.
Antiangiogenic therapies are significantly burdened by treatment-related adverse events. The overall incidence in the SHARP trial was 80% in the sorafenib-group and 52% in the placebo-group, mainly gastrointestinal and dermatologic events or constitutional symptoms.\textsuperscript{2} Furthermore, an upregulation of alternative pro-angiogenic signalling circuits is possible during the treatment. Even in presence of an initial response, the tumour vasculature may be restored, indicative of a re-initiation of tumour angiogenesis. Interestingly, in a mouse model of pancreatic neuroendocrine cancer, higher levels of mRNA for pro-angiogenic factors were found in relapsing tumours than in controls, suggesting the occurrence of resistance to or evasion of the anti-VEGF treatment.\textsuperscript{25} These factors, beyond the high costs of such drugs, encourage the search for a marker of tumour response in order to early switch the patients to more effective therapeutic regimens.

In our experiment, treated tumours showed an early increase in elasticity compared to vehicles, probably related to angioarchitectural changes inside the tumour. A possible underlying reason may be a reduction in tumour perfusion during the treatment. Indeed, a relative decrease in liquid content allows a higher deformation of a mass, which leads to a higher elasticity. Among treated tumours, we noted that non responders showed a different behaviour over time with respect to responders, that is higher elasticity at D0 and lower elasticity at D2. A possible explanation may be a strong cellularity before the treatment (responsible of higher elasticity values), then relatively decreased during the first days of treatment, and successively gone back to high levels.

The slight decrease over time of elasticity in the vehicle group may be the expression of the progressive tumorigenesis process. Indeed, newly formed vessels are characteristically dilated and microhemorrhaging, a condition that may result in a decrease of elasticity caused by the lower deformation of fluids if placed in a container.

The discrimination between responder and non-responder tumours is supported by western-blot analysis, which confirmed the higher VEGFR2 and phospho-ERK values in non responders.
compared to responders. Even if the statistical significance was not reached, a higher expression of phospho-ERK and VEGFR2 may reflect the activation of this pathway of cell proliferation even in presence of sorafenib. Furthermore, tumour volumes at day +14 were comparable between the non responder and the vehicle group.

The variability of treatment response in our work is related to the genetic type of the animal strain used. Indeed, we preferred to perform our experiment on outbred mice who are tipycally characterized by a high degree of heterozygosity and heterogeneity. Our results need to be validated in clinics. Being a proof of concept study, we decided to use a heterotopic model in order to avoid any confounding factor derived from the small dimension of the murine liver with respect to the probe and from the manual compression. Indeed, the compression on humans is performed through the intercostals spaces, a condition impossible to be created in a murine model using the commercially available elastonography probes.

In conclusion, we supported the use of elastosonography in the early evaluation of tumour response to antiangiogenic treatments, proving that responder tumours are characterized by an early increase in elasticity. The possibility to distinguish a priori between responders and non responders based on the higher elasticity of the latter needs to be validated in ad-hoc experiments as well as a confirmation of our results in humans is warranted.
BIBLIOGRAPHY


