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Laser speckle contrast imaging, the future DBF imaging technique for TRP target engagement biomarker assays

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ABSTRACT

A comparison was made between the established laser Doppler imaging (LDI) technique and the more recently developed laser speckle contrast imaging (LSCI) method to measure changes in capsaicin- and cinnamaldehyde-induced dermal blood flow (DBF) as an indicator of TRPV1 and TRPA1 activation, respectively.

Methods: Capsaicin (1000 μg/20 μl) and cinnamaldehyde (10%) solutions were applied on the forearm of 16 healthy male volunteers, alongside their corresponding vehicle solutions. Pre challenge and 10, 20, 30, 40 and 60 min post challenge application, changes in DBF were assessed with the LSCI technique, followed by LDI. The area under the curve from 0 to 60 min ($AUC_{0–60}$) post capsaicin and cinnamaldehyde application was calculated as a summary measure of the response. Correlation between the LDI and LSCI instrument was assessed using a simple linear regression analysis. Sample size calculations (SSC) were performed for future studies using either the LDI or LSCI technique.

Results: Higher arbitrary perfusion values were obtained with LDI compared to LSCI, yet a complete discrimination between the challenge and vehicle responses was achieved with both techniques. A strong degree of correlation was observed between LDI and LSCI measurements of the capsaicin- ($R = 0.84$ at Tmax and $R = 0.92$ for $AUC_{0–60}$) and cinnamaldehyde-induced ($R = 0.78$ at Tmax and $R = 0.81$ for $AUC_{0–60}$) increase in DBF. SSC revealed that LSCI requires considerably less subjects to obtain a power of 80% (about 15 versus 27 subjects in case of capsaicin and 7 versus 13 for cinnamaldehyde).

Conclusions: The LSCI technique was identified as the preferred method to capture capsaicin- and cinnamaldehyde-induced changes in DBF. Besides its reduced variability, the shorter scan time provides a major advantage, allowing real-time DBF measurements.

1. Introduction

Non-invasive imaging of superficial tissue perfusion is important in both clinical and research environments. First of all, the technique represents a valuable asset to monitor vascular activity in healthy or diseased tissue. For example, imaging the level of perfusion in burned tissue provides a rapid and reliable way to assess the depth of a burn, without the need of direct contact with the tissue (Stewart et al., 2005). Other applications include measuring blood flow changes in the brain of animal models of stroke and quantifying vascular changes in patients with primary and secondary Raynaud's phenomenon (Boas and Dunn, 2010; Murray et al., 2004). Besides, the technique can be used to evaluate blood flow responses after applying a stimulus, for instance to monitor the vasodilatory effect associated with local skin heating. Additionally, measuring changes in dermal blood flow (DBF) provides a non-invasive way to assess target engagement in vivo, in humans, with minimal risk for the subject. Over the years we have developed several of such “target engagement biomarkers” for the transient receptor potential (TRP) ion channel superfamily; appealing targets for innovative analgesic drugs. The principle behind these models is that the topical application of a selective agonist will activate the specific TRP channel, expressed on peripheral nerve endings of sensory neurons innervating the skin. As a result, the TRP nociceptor depolarizes, initiating action potentials that are conducted in both the orthodromic and antidromic direction. This antidromic impulse initiates the local release of different neural mediators from the nerve terminals, which in turn act on

Abbreviations: AUC, Area under the curve; CA, Cinnamaldehyde; CAPS, Capsaicin; CV, Coefficient of variation; DBF, Dermal blood flow; LDI, Laser Doppler imaging; LSCI, Laser speckle contrast imaging; PU, Perfusion unit; ROI, Region of interest; RMSE, Root mean square error; SEM, Standard error of the mean; SSC, Sample size calculations; TRPA1, Transient receptor potential Ankyrin 1; TRPV1, Transient receptor potential Vanilloid 1

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multiple neighboring cells, including vascular smooth muscle cells to produce vasodilation. Measuring these changes in DBF provides a non-invasive approach to evaluate TRP activation in vivo, in human (Buntinx et al., 2015). Capsaicin is exploited to evaluate TRP vanilloid 1 (TRPV1) target engagement, while cinnamaldehyde is used as a selective agonist for TRP ankyrin 1 (TRPA1) (Buntinx et al., 2017; Van der Schueren et al., 2007). Target engagement biomarker assays provide a valuable asset to support early clinical drug development, especially in therapeutic areas where drug attrition is high, with central nervous system diseases as a frontrunner. By evaluating target engagement early-on in clinical development, one can establish confidence in the drug candidate to guide go/no-go decisions and therefore accelerate the drug development process (Buntinx et al., 2015).

Both TRP target engagement biomarker models were validated with laser Doppler imaging (LDI), a technique that has gained interest as a method to monitor tissue perfusion since Stern's initial publication in 1975 (Stern, 1975). In the LDI technique, a low power laser beam is used to scan multiple points across a tissue surface. Due to the interaction of the laser with moving red blood cells in the tissue, light scatters and shifts wavelength, known as the Doppler effect. Part of the backscattered light is detected by a photodetector within the instrument and then converted into an electrical signal. This information is used to shape a two dimensional color coded perfusion image of the tissue scanned as the magnitude of the Doppler signal is related to the red blood cell concentration and the frequency shift can be linked to the mean velocity of moving erythrocytes. Spatial resolution is good for LDI but temporal resolution is rather poor as it takes 4 to 5 min to scan an area of about 12 × 12 cm (Karin Wårdell, 1992; Millet et al., 2011; Stewart et al., 2005). Temporal resolution is greatly improved in the more recently developed laser speckle contrast imaging (LSCI) technique, also referred to as laser speckle contrast analysis, which allows real time perfusion images at video rates (Briers et al., 2013). When a laser is used to illuminate a rough surface such as skin, the high coherence of the light generates a random interference pattern, termed speckle. If the illuminated object does not move, the speckle pattern will remain static in time. On the other hand, moving red blood cells in the illuminated tissue will shift the scattered light, thereby creating dynamic interference patterns, varying over time (Boas and Dunn, 2010; Briers, 2007; Stewart et al., 2005). Analogous to LDI, LSCI generates blood flow maps of the illuminated tissue. Nevertheless, limited data are available linking LSCI measurements to the actual blood flow in the tissue, whereas it is well known that there is a linear relationship between the LDI response and underlying tissue perfusion. In 2005, Stewart et al. demonstrated an excellent correlation (R^2 = 0,86 with n = 63) between the two techniques when evaluating burn scar perfusion (Stewart et al., 2005) and several years later, Millet et al. were the first to show a strong positive correlation (R = 0,90 with n = 12) in healthy skin after heat provocation (Millet et al., 2011). However, to date it is not clear if LDI and LSCI are equally suited to assess the DBF responses following capsaicin or cinnamaldehyde provocation as a way to evaluate target engagement. Moreover, in clinical trials validation is of critical importance to obtain accurate and precise measurements. Therefore, this study was set up to validate our target engagement biomarker models using the LSCI technique compared to LDI as the reference.

2. Methods

2.1. Subjects and study design

The study was conducted in accordance with the latest version of the Declaration of Helsinki and International Guidelines on Clinical Trials of Medicinal Products (ICH/GCP Topic E6-July 1996). Approval was obtained from the Ethics Committee of the University Hospitals Leuven, Leuven, Belgium (S57829) as well as the Federal Agency for Health and Medicinal Products (FAHMP) of Belgium (EudraCT 2014-004736-19).

The study design as well as in- and exclusion criteria were adopted from earlier studies in which the capsaicin (CAPS) and cinnamaldehyde (CA) target engagement biomarker models were validated (Buntinx et al., 2017; Van der Schueren et al., 2007). A total of sixteen subjects was recruited through an approved database available at the Center for Clinical Pharmacology. Written informed consent was obtained from each subject before participation. Upon arrival, subjects were sited in a temperature controlled room (23 °C ± 1 °C), where all measurements took place. First, study restrictions were checked and vital parameters were collected. All subjects were healthy (based on medical history, body mass index (BMI) and vital signs), Caucasian, non-smoking males with age limits between 18 and 45 years. Volunteers with a skin disorder, excessive forearm hair growth or allergies to any of the investigational products were excluded. Furthermore, subjects were not allowed to take any medication in a period of 14 days preceding the study visit. Besides, subjects were instructed to refrain from alcohol and caffeine consumption 24 h before the study visit and to avoid applying topical treatments or lotions on their forearms. Fasting was demanded 3 h prior to the study visit. As part of the vital signs, subject's weight, height, tympanic temperature (Genius™ 2 tympanic thermometer, Covidien), blood pressure and heart rate (Omron® 705IT, digital automatic blood pressure monitor, Intellisense™) were measured.

After a 30 min acclimatization period in a recumbent position, the subject's forearms were placed in U-shaped cushions to avoid movement. Three 10 mm rubber O-shaped rings (Quad Ring BS011 NBR 70 Shore A, Polymax Ltd) were placed on the volar surface of both forearms, at least 5 cm away from the elbow and wrist, avoiding visible veins. Pre challenge DBF measurements were performed prior to the application of a 20 µl topical dose of CA (2 µl/20 µl) and CAPS (1000 µg/20 µl) in the two most proximal rings on the right and left forearm, respectively. The distal ring on both arms was reserved for a 20 µl topical dose of vehicle. Next, the DBF in each ring was imaged with LSCI, followed by LDI approximately 10, 20, 30, 40 and 60 min post application. Only subjects with a CA- and CAPS-induced increase in DBF of 100% or more compared to the pre challenge measurement were included.

2.2. Cinnamaldehyde and capsaicin solutions

Trans-cinnamaldehyde oil 99% (Sigma-Aldrich, Bornem, Belgium) was dissolved in a 3:3:1 (v:v) mixture of ethanol 96% denatured, polysorbate 20 and purified water to achieve a 10% (2 µl/20 µl) CA concentration. The vehicle solution matched the same 3:3:1 mixture of ethanol 96% denatured, polysorbate 20 and purified water, lacking CA (Buntinx et al., 2017).

Capsaicin powder ≥ 95% (Sigma-Aldrich, Bornem, Belgium) was dissolved in a 3:3:4 (w:v) mixture of ethanol 96% denatured, polysorbate 20 and purified water to achieve a 1000 µg/20 µl CAPS concentration. The vehicle solution matched the same 3:3:4 mixture of ethanol 96% denatured, polysorbate 20 and purified water, lacking CAPS (Van der Schueren et al., 2007).

2.3. LDI and LSCI instruments

DBF was measured pre and 10, 20, 30, 40 and 60 min post application of CA, CAPS or vehicle. At each time point, DBF was first measured with the LSCI instrument (PeriCam PSI system, Perimed, Järfälla, Sweden), followed by LDI (PeriScan PIM 3 system, Perimed, Järfälla, Sweden). In case of the LSCI system, a 785 nm laser was used to illuminate the tissue with an exposure time of 6 ms. The laser head was positioned 25.0 ± 1.0 cm above the surface of the subject's forearm. A surface area of 15.0 by 20.0 cm was selected for the scan size, incorporating the three rubber rings in one perfusion image. Duration was set on a number of five images, using an acquisition rate of 0.5 images per second. The perfusion scale was determined automatically with an

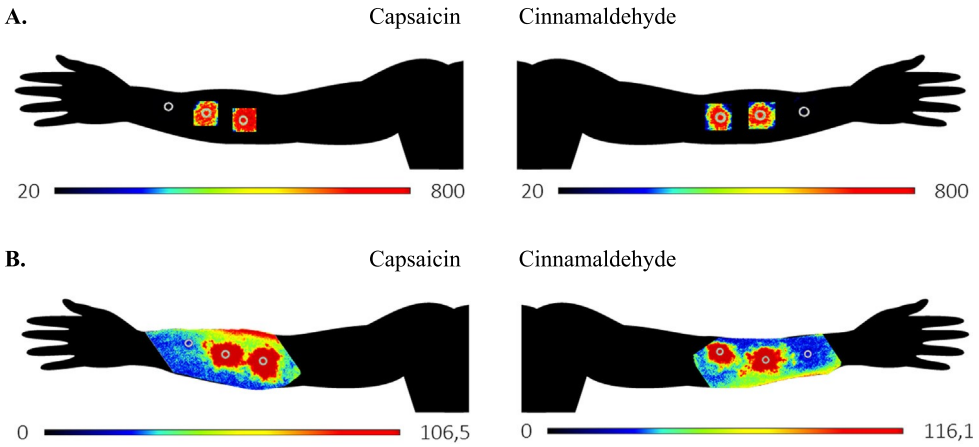

Fig. 1. LDI and LSCI images of the cinnamaldehyde- and capsaicin-induced DBF: Representative images of the CA- and CAPS-induced DBF,measured 20 and 40 min after topical application on the right and left forearm, respectively, in one subject (n = 1). A. LDI requires time to scan the skin point by point. Therefore, three small scan areas (3.0 × 3.0 cm) were measured, one for each ring. The perfusion scale was fixed at 20 to 800 PUs. B. LSCI can instantly measure DBF over a larger tissue area (15.0 × 20.0 cm), incorporating the three rubber rings. The perfusion scale was computed automatically.

intensity filter of 0.14 to 10.00.

The LDI system incorporates a low power 658 nm laser. The head of the scanner was placed 20.0 ± 1.0 cm above the skin surface with an image size of 3.0 × 3.0 cm. Each application area was measured separately, requiring three images per arm at each time point. A high point density was selected and the duration was set on snapshot. A perfusion scale of 20 to 800 perfusion units (PUs) was chosen with an intensity filter of 0.37 to 10.00.

2.4. Data analysis and statistics

Demographic information was analyzed using descriptive statistics and expressed as mean ± SD (range). Both LDI and LSCI data were processed using PIMSoft software (PIMSoft, Perimed, Järfälla, Sweden). LSCI perfusion images were first averaged, using a reduction factor of 5, to create one representative image for each time point. Next, regions of interest (ROI) were selected within the print of the 10 mm (8 mm inner diameter) O-shaped rings, adjacent to the inside border. The mean perfusion within each ROI was calculated using PIMSoft. The software expresses the recorded perfusion of both imaging techniques in arbitrary perfusion units (PUs), which corresponds to the concentration of red blood cells times their average velocity.

To obtain the CA- and CAPS-induced DBF response, the mean perfusion within the two proximal rings on the right and left forearm, respectively, was averaged. The pattern of change in DBF over time was acquired by averaging the perfusion values of all subjects at each time point for both techniques. This mean DBF was then plotted together with the standard error of the mean (SEM), while the area under the curve from 0 to 60 min ($AUC_{0–60}$) post CAPS and CA application was calculated as a summary measure of the response. Analysis was performed by paired-samples *t*-testing with a *p*-value < 0.05 considered statistically significant. The correlation between the LDI and LSCI instrument was assessed using a simple linear regression analysis on the cutaneous perfusion at Tmax of the challenges as well as on the $AUC_{0–60}$ values measured with both techniques. Besides the Pearson correlation coefficient R ± 95% confidence interval (CI), also the root mean square error (RMSE) was calculated. Finally, sample size calculations (SSC) were performed for a paired samples t-test using PS: Power and Sample Size Calculator® software. Sample sizes were calculated in order to detect a shift of 10, 20, 30 and 50% in either the DBF response at Tmax or the $AUC_{0–60}$, for both LDI and LSCI, given a significance level of 5% and a power of 80%.

3. Results

3.1. Demographics

In total, twenty-two subjects were screened of whom two suffered from eczema, two used concomitant medication, one BMI was too high and one subject did not respond to capsaicin. Ultimately, sixteen non-smoking, Caucasian males were included. Mean ± SD (range) were 23 ± 4 (18–36) years for age, 23 ± 2 (20–26) kg/m^2 for BMI, 129 ± 7 (119–139) mmHg for systolic and 72 ± 5 (62–82) mmHg for diastolic blood pressure and 61 ± 15 (39–95) bpm for heart rate. All included subjects completed the study.

3.2. LDI and LSCI measurements of the cinnamaldehyde- and capsaicin-induced DBF

When stimulating TRPV1 and TRPA1 with CA and CAPS respectively, several vasoactive mediators are released, causing an increase in DBF. These DBF changes were measured with LDI and LSCI during the hour following topical application of the agonists. LSCI instantly captured snapshots of the subject's forearm, while LDI had to take three images per arm, scanning the 3.0 × 3.0 cm tissue area stepwise, thereby requiring on average ± SD (range) 41 ± 3 (36–51) seconds per image. For LDI the perfusion scale was set at 20 to 800 PUs, while for the LSCI instrument this scale was determined automatically, starting at 0 up to 103 ± 15 (81–127) PUs for CA and from 0 to 94 ± 8 (78–114) PUs for CAPS. Fig. 1 shows typical LDI and LSCI images of one healthy volunteer ($n = 1$), 20 and 40 min post CA and CAPS application respectively.

As the difference in perfusion scales already suggests, higher arbitrary perfusion values were obtained with LDI compared to LSCI. At the Tmax of CAPS, 40 min following topical application, LDI measured a DBF of 457 ± 162 (276–796) PUs with a coefficient of variation (CV) of 35%, while LSCI only obtained 114 ± 31 (75–198) PUs with a CV of 27%. At the Tmax of CA, 20 min after its application, measuring with LDI resulted in a perfusion value of 445 ± 110 (287–731) PUs with a CV of 25% compared to 123 ± 24 (92–195) PUs for LSCI, with a CV of 19%. Nonetheless, both techniques revealed a similar pattern of change in DBF over time (Fig. 2).

Despite the lower DBF values obtained with the LSCI instrument, challenge and vehicle responses are completely separated using either technique (Fig. 3).

3.3. Correlation between LDI and LSCI measurements of the cinnamaldehyde- and capsaicin-induced DBF

Simple regression analyses on the DBF values obtained with LDI and LSCI at the Tmax of CA ($R = 0.78$; Fig. 4A) and CAPS ($R = 0.84$; Fig. 4B) revealed a strong linear correlation between the two techniques with RMSE values of 15.2 and 17.4 for CA and CAPS, respectively.

Likewise, a very good correlation between LDI and LSCI was found when comparing the $AUC_{0–60}$ after CA ($R = 0.81$; Fig. 4C) and CAPS ($R = 0.92$; Fig. 4D) application, with an RMSE value of 660.2 in the

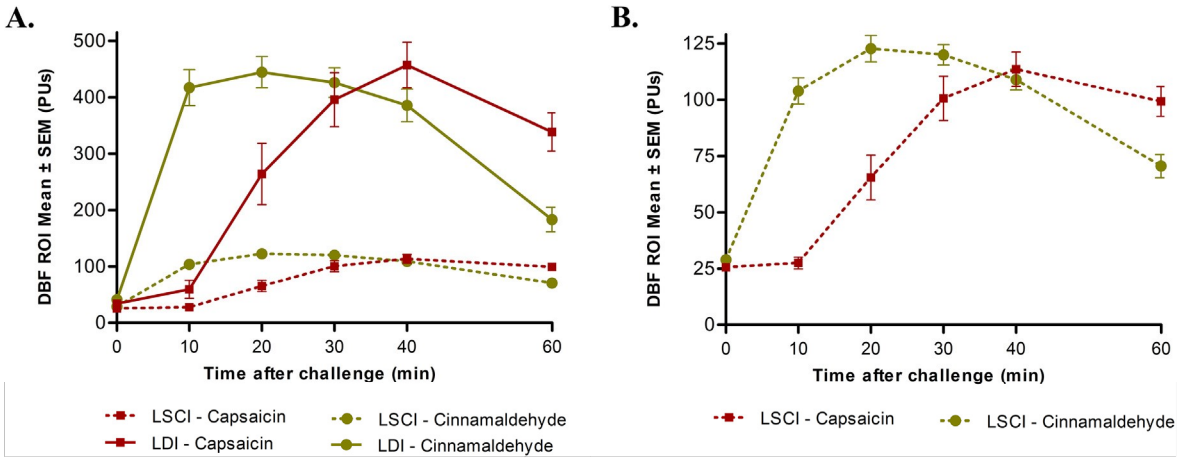

Fig. 2. DBF changes over time following topical application of cinnamaldehyde and capsaicin, obtained with LDI and LSCI: CA and CAPS were applied on the volar surface of subject's forearm ($n = 16$) after which the DBF was measured at 10, 20, 30, 40 and 60 min. The changes in DBF follow a similar time course for LDI and LSCI, but LSCI records lower perfusion values. A. CA- and CAPS-induced changes in DBF ± SEM measured with LDI and LSCI. B. Close-up of CA- and CAPS-induced DBF changes ± SEM measured with LSCI.

case of CA and 564.8 for CAPS.

3.4. Sample size calculations (SSC)

SSC demonstrated that LSCI requires considerably less subjects to detect a change of 10% up to 50% in DBF between two independent groups, both at Tmax of the response (Table 1) and for $AUC_{0–60}$ as a summary measure (Table 2).

4. Discussion

In central nervous system diseases, where drug attrition is high, target engagement biomarkers provide a valuable asset to support early clinical drug development. An ideal target engagement biomarker enables an objective read-out of the interaction between a drug and its target, is non-invasive, safe and easy-to-use. LDI and LSCI are two non-invasive methods to determine cutaneous blood flow that raise minimal risk or discomfort for the subject. Although both techniques are based on the same physical principle, they approach the phenomenon differently. LDI calculations employ the power spectrum of Doppler shifts originating when laser light interacts with moving red blood cells, while LSCI analyses reductions in speckle contrast (Fredriksson and Larsson, 2016). Laser Doppler systems have been around for over two decades, whereas LSCI was only recently commercialized (Essex and Byrne, 1991; Wårdell et al., 1993). Contrary to the changes in Doppler frequencies, speckle contrast is not proportional to the speed of moving red blood cells and as such, a theoretical model linking LSCI measurements to actual perfusion values is difficult to establish (Briers et al., 2013; Fredriksson et al., 2019; Fredriksson and Larsson, 2016; Humeau-Heurtier et al., 2013; O'Doherty et al., 2009). The present study aimed to assess the ability of the LSCI technique to evaluate TRPV1 and TRPA1 activation, compared to LDI as the validated standard.

A first alteration originating from the different LDI and LSCI imaging techniques is the spatial and temporal resolution of the instruments. LDI achieves a two dimensional perfusion image by scanning the tissue surface in a stepwise fashion, temporarily halting to perform calculations. Therefore the acquisition time is long, on average 41 s for a 3.0 × 3.0 cm tissue area, which induces an inevitable time bias between the measured points. LSCI has the advantage of acquiring all measurements in parallel, allowing real-time DBF images and even video-recordings. This fast temporal response is especially interesting when monitoring dynamic DBF changes in response to pharmacological interventions, as is the case in our CA and CAPS target engagement biomarker models. Nevertheless, the weakness associated with these ultra-fast LSCI measurements is a higher susceptibility to movement artifacts, even though both LDI and LSCI are very sensitive to movement. All subjects were asked to lie as still as possible during the DBF measurements, but even involuntary movements like twitching and breathing motions may affect the near instantaneous snapshots

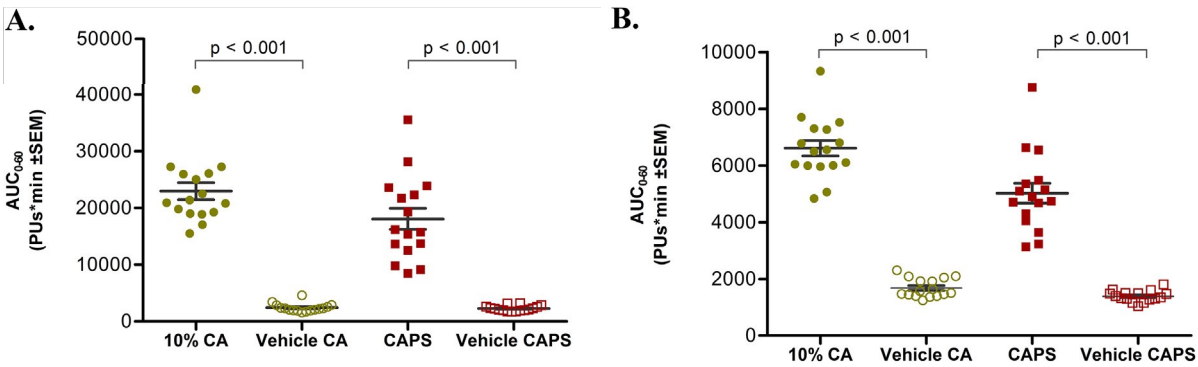

Fig. 3. Cinnamaldehyde- and capsaicin-induced DBF responses measured with LDI and LSCI: The $AUC_{0–60}$ after topical application of CA and its vehicle as well as CAPS and its vehicle were calculated as a summary measure (n = 16). For both imaging techniques, a complete separation between challenge and vehicle was observed. Paired-samples *t*-test revealed a significant difference ($p < 0.001$) between challenge and vehicle responses for both techniques. A. $AUC_{0–60}$ calculated for the LDI instrument. B. $AUC_{0–60}$ calculated for the LSCI instrument.

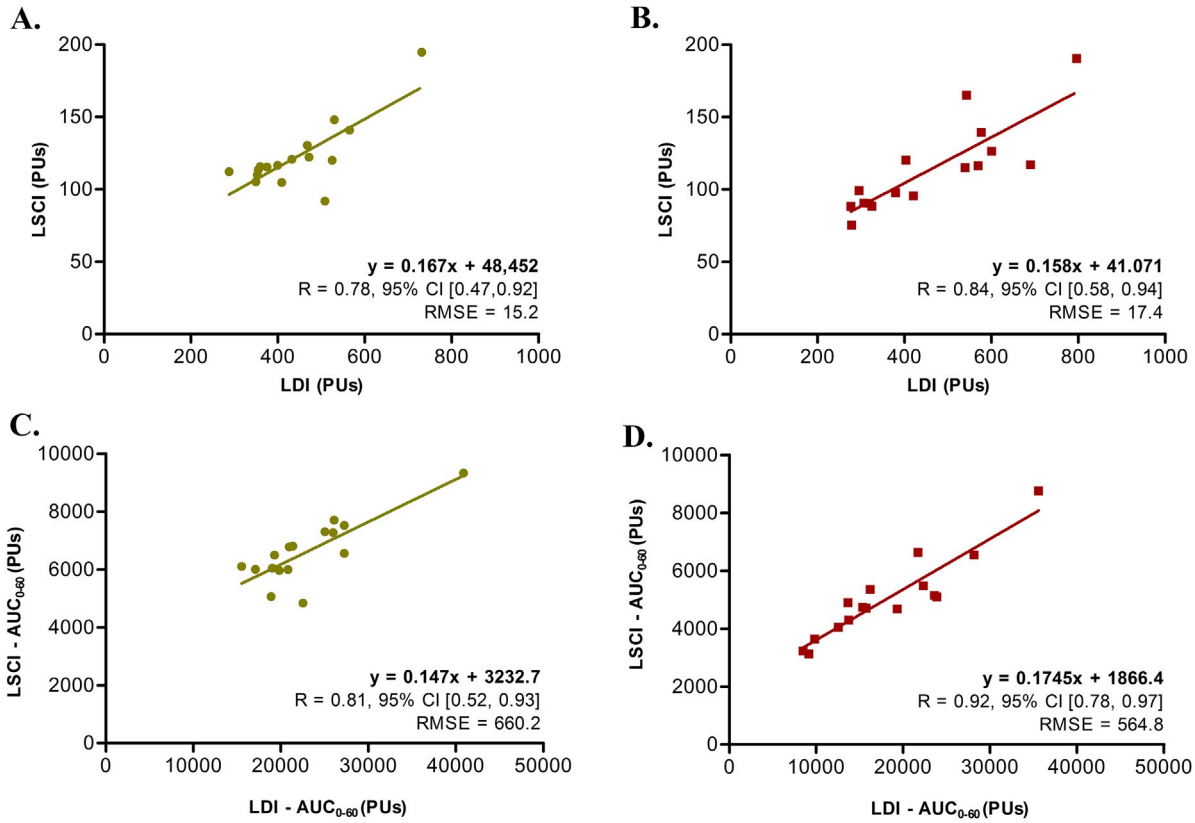

Fig. 4. Correlation between LDI and LSCI measurements of the cinnamaldehyde- and capsaicin-induced DBF at Tmax and expressed as AUC: CA and CAPS were applied on the volar surface of the subject's forearm (n = 16) after which the DBF was measured at 10, 20, 30, 40 and 60 min. A simple regression analysis was performed to assess the correlation between LDI and LSCI, together with the Pearson correlation coefficient R and root mean square error (RMSE). A. Correlation between the CA-induced DBF measured with LDI and LSCI, 20 min after CA application. B. Correlation between the CAPS-induced DBF measured with LDI and LSCI, 40 min after CAPS application. C. Correlation between the $AUC_{0–60}$ after CA application, obtained with LDI and LSCI. D. Correlation between $AUC_{0–60}$ after CAPS application, obtained with LDI and LSCI.

Table 1
Sample size calculations for cinnamaldehyde- and capsaicin-induced DBF at Tmax: For both the LDI and LSCI instrument, the required sample size was calculated to detect a difference of 10, 20, 30 and 50% between two independent groups with a significance level of 5% and power of 80%.

Instrument – challenge (Tmax)	SSC 10% difference	SSC 20% difference	SSC 30% difference	SSC 50% difference
LDI – CA (20 min)	50	14	7	4
LSCI – CA (20 min)	31	9	5	3
LDI – CAPS (40 min)	100	26	13	6
LSCI – CAPS (40 min)	59	16	8	4

Table 2
Sample size calculations for the $AUC_{0–60}$ after cinnamaldehyde and capsaicin application: For both the LDI and LSCI instrument, the required sample size was calculated to detect a difference of 10, 20, 30 and 50% between two independent groups with a significance level of 5% and power of 80%.

Instrument - challenge	SSC 10% difference	SSC 20% difference	SSC 30% difference	SSC 50% difference
LDI – CA	55	15	8	4
LSCI – CA	23	7	5	3
LDI – CAPS	135	35	17	7
LSCI – CAPS	64	17	9	5

captured with LSCI. In this protocol, movement artifacts were kept to a minimum by executing five consecutive images at each time point in order to obtain one averaged, representative LSCI measurement. Besides, an opaque surface close to the measurement area can be used to correct for motion artifacts (Mahé et al., 2011). Anyhow, to fully enjoy the exciting possibility of real-time perfusion measurements using LSCI, it is important to be aware of and control for possible movement artifacts.

A second difference concerns the measurement depth of the instruments in the skin. Although there is no consensus on the precise depth of the dermal blood flow measurements in the forearm, it is generally accepted that LSCI penetrates the tissue less deep (approximately 300 μm) than the LDI technique (circa 500–1000 μm). As a consequence, speckle recordings only capture the superficial subpapillary plexus of the skin, instead of the more ascending arterioles that can be reached using LDI. This is the drawback associated with full-field imaging as the LSCI laser source is stretched over a larger tissue area, reducing the power density of the laser beam and thereby losing information from deeper vessels (Humeau-Heurtier et al., 2013; O'Doherty et al., 2009). In our target engagement biomarker models, skin penetration of CA and CAPS is presumed limited as we aim for a local effect on the TRP channels expressed on sensory neurons innervating the skin. Therefore, imaging capillary flow should be sufficient to demonstrate our challenge effect. Nonetheless, as LDI and LSCI do not measure perfusion from exactly the same skin layers, some variation on the obtained DBF values was expected. Still, a strong correlation between the two imagers (R = 0.78/0.84 at the Tmax of CA/CAPS and R = 0.81/0.92 for the $AUC_{0–60}$ of CA/CAPS) was observed. This perceived association is presumably even a slight

underestimation of the true correlation due to the time difference between LSCI and LDI measurements. As it was not possible to measure DBF with both instruments simultaneously, LSCI measurements were performed first, followed by the time-consuming LDI technique. Though the induced time bias is considered small, a minor effect on the instruments' relationship can be expected. This may explain partly why the correlation detected in this study is less than the ones observed by Stewart and colleagues in determining burn scar perfusion ($R^2 = 0.86$) and by Millet et al. who applied local skin heating as DBF stimulus ($R = 0.90$). However, also the specific stimuli used in the present study are likely to play a role. As the vasodilatory effect evoked by CA and CAPS is rather strong, red blood cells move with high velocity through the arteries. At those high speeds, LSCI is presumably less sensitive using a single exposure time while LDI measures the velocity of moving red blood cells at different time points. Furthermore, the nonlinear relation between LSCI contrast and blood flow speed is dependent on the concentration of red blood cells (Fredriksson and Larsson, 2016). Therefore, the instruments correlate better at lower red blood cell velocities, as also illustrated by the higher Pearson correlation coefficients obtained for the $AUC_{0–60}$ as a summary measure. Nevertheless, even in the case of high skin perfusion, LDI and LSCI DBF measurements correlate strongly.

It is important to note that neither the laser Doppler, nor the speckle contrast signal is capable of generating absolute perfusion measurements. Both instruments require calibration in order to compare the flow output (Briers et al., 2013; Rajan et al., 2009). As such, DBF is reported in arbitrary perfusion units, yet scaling is different. LDI consistently reports higher perfusion values, hampering a direct comparison with LSCI measurements. Therefore, a simple linear regression analysis was used to enable an association between newly obtained LSCI perfusion values and the LDI measurements reported in earlier studies. Although only based on a limited sample of the population, the MRSE values associated with these regression equations indicate that the error is reasonable when using the obtained formula to easily predict LSCI DBF values from observed LDI measurements. More precise, yet more demanding calculations have been described elsewhere (Fredriksson et al., 2019; Fredriksson and Larsson, 2016), but our easy to use equations will certainly smoothen the transition from LDI to LSCI in clinical trials. However, besides hampering a direct comparison with LDI measurements, it remains to be shown whether the lower perfusion values obtained with LSCI are sufficient to employ as a target engagement biomarker for possible TRP antagonists. It should be noted that inhibition of the LSCI DBF response was not evaluated as part of this paper. This could be an interesting suggestion for future research. Yet, the complete separation of the agonist-induced DBF response and the corresponding vehicle already hints that the increase in DBF measured with LSCI should be sufficient to employ as a target engagement biomarker.

Finally, the major advantage associated with the LSCI instrument is its reduced variability as the technique consistently delivered lower inter-subject coefficients of variation (27% versus 35% in the case of CAPS and 19% versus 25% for CA). As a result, LSCI requires considerably less subjects than the LDI instrument to obtain a power of 80%. Using the LSCI technique, a minimum of five subjects should suffice to detect a difference of 50% in the CAPS-induced DBF between paired groups. The CA target engagement biomarker model is even more repeatable, requiring only four subjects to identify a difference of 50%. Noteworthy, this discrepancy between the CAPS and CA response is not a direct effect of the LSCI imaging method as in general the CAPS response is known to be more variable among subjects (Buntinx et al., 2017; Van der Schueren et al., 2007). Nevertheless, the lower variability obtained with the LSCI technique paves the way for interesting clinical applications. For instance, Ibrahimi et al. were only able to detect a menstrual cycle dependence in the CAPS-induced DBF response of healthy women, not in female migraine patients (Ibrahimi et al., 2017). This finding was somewhat unexpected and may be explained by the heterogeneity of the disorder. Therefore, it cannot be excluded that LDI, being the more variable DBF imaging technique, was not able to distinguish these subtle differences in CAPS-induced DBF responses. Repeating the study with LSCI might provide an interesting new perspective. Nonetheless, it should be noted that the present study only validated LSCI as a DBF read-out in healthy male volunteers as the study sample was based on the typical subjects included in a phase I clinical trial, in which the target engagement biomarker models are generally used. Validating the LSCI instrument in a broader, more diverse population, for example migraine patients to repeat the study of Ibrahimi et al., could be an interesting topic for future research.

In conclusion, the present study validated the use of LSCI as a DBF read-out in the CAPS and CA target engagement biomarker models. A strong correlation between LDI and LSCI DBF measurements was observed, but higher perfusion values were obtained using the LDI technique. Nonetheless, a complete separation between challenge and vehicle responses was achieved for both techniques. Interestingly, LSCI proved to be the less variable imaging method, resulting in smaller sample sizes required to detect statistically significant changes in perfusion. Moreover, its fast temporal response is advantageous when monitoring dynamic DBF changes. As such, the more recent LSCI instrument was identified as the preferred method to evaluate TRP activation, compared to the established LDI technique.

CRediT authorship contribution statement

Dorien Bamps: Data acquisition, Formal analysis, Investigation, Writing - original draft, Visualization. **Laura Macours:** Data acquisition, Investigation, Writing - review & editing. **Linde Buntinx:** Conceptualization, Writing - review & editing. **Jan de Hoon:** Conceptualization, Writing - review & editing, Supervision.

Declaration of competing interest

The authors have no conflict of interest to declare. This research was funded by a KU Leuven Belgium Internal Fund (3M170309).

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