

# Single-cell analysis of $[Ca^{2+}]_i$ signalling in subfertile men: characteristics and relation to fertilisation outcome

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**Single-cell analysis of  $[Ca^{2+}]_i$  signalling in subfertile men: characteristics and relation to fertilisation outcome**

**Running Title:**  $[Ca^{2+}]_i$  signalling in spermatozoa of sub-fertile men

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## 17 **Abstract**

18 **Study question:** What are the characteristics of progesterone-induced (CatSper-mediated)  
19 single cell  $[Ca^{2+}]_i$  signals in spermatozoa from sub-fertile men and how do they relate to  
20 fertilising ability?

21 **Summary answer:** Single cell analysis of progesterone-induced (CatSper-mediated)  $[Ca^{2+}]_i$   
22 showed that reduced progesterone-sensitivity is a common feature of sperm from sub-  
23 fertile patients and is correlated with fertilization rate.

24 **What is known already:** Stimulation with progesterone is a widely-used method for  
25 assessing  $[Ca^{2+}]_i$  mobilisation by activation of CatSper in human spermatozoa. Although data  
26 are limited, sperm population studies have indicated an association of poor  $[Ca^{2+}]_i$  response  
27 to progesterone with reduced fertilization ability.

28 **Study design, size, duration:** This was a cohort study using semen samples from 21 donors  
29 and 101 patients attending the assisted conception unit at Ninewells Hospital Dundee who  
30 were undergoing ART treatment. Patients were recruited from January 2016-June 2017.

31 **Participants/materials, setting, methods:** Semen donors and patients were recruited in  
32 accordance with local ethics approval (13/ES/0091) from the East of Scotland Research  
33 Ethics Service (EoSRES) REC1.  $[Ca^{2+}]_i$  responses were examined by single cell imaging and  
34 motility parameters assessed by computer-assisted sperm analysis (CASA).

35 **Main results and the role of chance:** For analysis, patient samples were divided into three  
36 groups IVF(+ve) (successful fertilisation; 62 samples), IVF-FF (failed fertilisation; 8 samples)  
37 and ICSI (21 samples). A further 10 IVF samples showed large, spontaneous  $[Ca^{2+}]_i$   
38 oscillations and responses to progesterone could not be analysed. All patient samples  
39 loaded with the  $[Ca^{2+}]_i$ -indicator fluo4 responded to progesterone stimulation with a  
40 biphasic increase in fluorescence (transient followed by plateau) which resembling that seen

in progesterone-stimulated donor samples. The mean normalized response (progesterone-induced increase in fluorescence normalized to resting level) was significantly smaller in IVF-FF and ICSI patient groups than in donors. All samples were further analysed by plotting, for each cell, the relationship between resting fluorescence intensity and the progesterone-induced fluorescence increment. In donor samples these plots overlaid closely and had a gradient of  $\approx 2$  and plots for most IVF(+ve) samples closely resembled the donor distribution. However, in a subset ( $\approx 10\%$ ) of IVF(+ve) samples, 3/8 IVF-FF samples and one third of ICSI samples the gradient of the plot was significantly lower, indicating that the response to progesterone of the cells in these samples was abnormally small. Examination of the relationship between gradient (regression coefficient of the plot) in IVF samples and fertilisation rate showed a positive correlation. In IVF-FF and ICSI groups, the proportion of cells in which a response to progesterone could be detected was significantly lower than in donors and IVF (+ve) patients. Approximately 20% of cells in donor, IVF(+ve) and ICSI samples generated  $[Ca^{2+}]_i$  oscillations when challenged with progesterone but in IVF-FF samples only  $\approx 10\%$  of cells generated oscillations and there was a significantly greater proportion of samples where no oscillations were observed. Levels of hyperactivated motility were lower in IVF(+ve) and IVF-FF groups compared to controls, IVF-FF also having lower levels than IVF(+ve).

**Limitations, reasons for caution:** This is an *in vitro* study and caution must be taken when extrapolating these results *in vivo*.

**Wider implications of the findings:** This study reveals important details of impaired  $[Ca^{2+}]_i$  signalling in sperm from sub-fertile men that cannot be detected in population studies

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66

67     **Key words:** Calcium signalling / CatSper channel / spermatozoa / subfertility / progesterone

## 68 Introduction

69 Sperm dysfunction is commonly regarded as the single most common cause of infertility yet  
70 there is a paucity of non-ART treatments available (Martins da Silva *et al.*, 2017). A detailed  
71 understanding of the working of the normal and dysfunctional cell is necessary to develop a  
72 platform for new diagnostic and treatment options (Barratt *et al.*, 2017, 2018). Intracellular  
73  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) signalling is fundamental in regulation of many aspects of sperm function  
74 including motility and the acrosome reaction (Publicover *et al.*, 2007) and dysregulation of  
75 any aspect of sperm  $[\text{Ca}^{2+}]_i$  signalling is thought to impair the normal function of sperm and  
76 reduce fertilisation capability (Krausz *et al.*, 1995; Williams *et al.*, 2015). CatSper, the  
77 primary  $\text{Ca}^{2+}$ -influx channel of sperm, is weakly voltage-sensitive and is activated by  
78 intracellular alkalinisation, but in human sperm is also sensitive to a variety of ligands, the  
79 best-characterised of which is progesterone (P4; Lishko *et al.*, 2011; Strunker *et al.*, 2011).  
80 P4 may therefore cause strong activation of the channel as sperm approach the oocyte, the  
81 consequent  $\text{Ca}^{2+}$  influx regulating activities required for fertilisation (Lishko *et al.*, 2012).  
82 Mouse sperm null for CatSper are sterile (Ren *et al.*, 2001) and previous studies on sperm  
83 from ART patients revealed impaired  $[\text{Ca}^{2+}]_i$  handling and reduced ability to respond to P4,  
84 particularly in samples that subsequently failed to fertilise at IVF, indicating that CatSper  
85 lesions may underlie reduced fertility in these men (Krausz *et al.*, 1995, 1996; Alasmari *et al.*,  
86 2013a) Recently Williams *et al.* (2015) combined screening of P4-induced  $[\text{Ca}^{2+}]_i$  signals  
87 with direct assessment of CatSper currents to show that a complete lack of functional  
88 CatSper (no  $[\text{Ca}^{2+}]_i$  response to P4 or membrane current) is enough to compromise fertilising  
89 ability and IVF outcome. Interestingly, though only one patient had no detectable CatSper  
90 function, several patients had more subtle abnormalities of the  $[\text{Ca}^{2+}]_i$  response when  
91 challenged with P4 (Williams *et al.*, 2015).

P4  $[Ca^{2+}]_i$  responses of individual sperm vary greatly within a single ejaculate (Kirkman-Brown *et al.*, 2000). For instance, within a sample the response to P4 of an individual cell may be negligible or may exceed modal amplitude by >2-fold (Kirkman-Brown *et al.*, 2000; Lefievre *et al.*, 2012). However, all previous studies on CatSper-mediated  $[Ca^{2+}]_i$  responses of ART patients have used fluorimetric techniques that record only the summed response of a large population (Krausz *et al.*, 1995, 1996; Williams *et al.*, 2015). Though showing clearly that  $[Ca^{2+}]_i$  signalling in sub-fertile men is abnormal, this approach provides no information on the distribution of single cell responses in these samples and how this varies compared to that of 'normal' (donor) cells.

Although time consuming and technically more complex, single cell  $[Ca^{2+}]_i$  imaging provides information on activity of individual sperm that cannot be obtained by studying populations, including the proportion of responsive cells, the presence of sub-populations that respond differently and the nature and complexity of the single cell  $[Ca^{2+}]_i$  signal. We have used single cell imaging to investigate responses to P4 in sperm samples from sub-fertile men attending an ART clinic, specifically 1) the nature and heterogeneity of single cell  $[Ca^{2+}]_i$  responses and 2) the relationship between P4-induced  $[Ca^{2+}]_i$  responses and fertilisation success.

## **Materials and Methods**

### **Experimental design**

Single cell  $[Ca^{2+}]_i$  imaging of spermatozoa from patients was carried out using an aliquot of the sperm preparation used for ART. Measurements were made on the day of treatment, allowing direct correlation with ART. Computer-assisted sperm analysis (CASA) was done on each aliquot. For analysis, patient samples were divided into three groups IVF(+ve) (successful fertilisation), IVF-FF (failed fertilisation) and ICSI.

### **Ethical approval**

Written consent was obtained from each patient in accordance with the Human Fertilization and Embryology Authority (HFEA) Code of Practice (version 8) under local ethical approval (13/ES/0091) from the Tayside Committee of Medical Research Ethics B. Similarly, volunteer sperm donors were recruited in accordance with the HFEA Code of Practice (version 8) under the same ethical approval.

### **Selection and preparation of spermatozoa**

Patients were selected for treatment according to clinical criteria and semen quality: i.e. those with normal sperm concentration and motility (WHO, 2010) and  $1 \times 10^6$  progressively motile cells post-preparation were selected for IVF, those who failed to meet these criteria were treated by ICSI. 441 patients attended the clinic and provided samples during the study period (January 2016-June 2017) of which 101 were tested. Supplementary Information Figure S7 presents the flowchart of patients and reasons for inclusion/exclusion. The surplus clinical sample used in the IVF/ICSI treatment was used where consent was given. Control semen samples were obtained from volunteer donors



with normal sperm concentration, motility and semen characteristics (WHO, 2010) and no known fertility problems. Samples were obtained by masturbation after 48–72 h of sexual abstinence.

Patient cells were prepared according to the standard operating procedures employed by the ACU and donor cells were prepared in an identical fashion but with equivalent bicarbonate buffered sperm capacitation medium prepared in house (Brown *et al.*, 2016). After 30 min of liquefaction at 37°C, donor and patient sperm were isolated using a discontinuous density gradient procedure (Tardif *et al.*, 2014; Williams *et al.*, 2015). Up to 2 ml of semen was loaded on top of a 40%-80% suspension of Percoll (Sigma Aldrich, UK;) diluted with HEPES buffered saline (donor semen) or Pureception (colloidal silica suspension for sperm preparation; Origio, Denmark) diluted with Spermwash (Origio, Denmark; patient semen). The density gradient was then centrifuged at 300 g for 20 min, washed (300 g, 10 min) and re-suspended in bicarbonate buffered sperm capacitation medium or Quinn's advantage human tubal fluid (HTF) (Origio, Denmark) (donor and patients respectively). All samples were left to capacitate at 37°C, 95% O<sub>2</sub>/5% CO<sub>2</sub> for a 5-7 hours prior to experimentation. Samples were obtained and analysed in line with suggested guidance for human semen studies where appropriate (Björndahl *et al.*, 2016). To assess whether [Ca<sup>2+</sup>]<sub>i</sub> responses were affected by preparation protocol, control experiments on donor cells were carried out in which semen samples were split and prepared in parallel as described above using IVF clinic medium for one aliquot and bicarbonate buffered sperm capacitation medium for the other. P4-induced Ca<sup>2+</sup> signals were similar in cells prepared by the two methods (Supplementary Information Figure S1).

## Single cell $[Ca^{2+}]_i$ imaging

Sperm were prepared and assessed as previously described (Brown *et al.*, 2017). Briefly, capacitated sperm (1-2 million cells/ml) were loaded with 2  $\mu$ M Fluo-4 (Molecular Probes, UK) at 37°C for 20 min then centrifuged at 300 g for 10 min. The supernatant was removed and pellet re-suspended in supplemented Earle's balanced salt solution (sEBSS). This wash step was repeated and the pellet was re-suspended in sEBSS for imaging. Sperm were loaded into a small-volume imaging chamber (RC-20, Harvard apparatus UK) sealed with vacuum grease (DowCorning 976) on a poly-D-lysine (0.05%) coated coverslip, and allowed to adhere for ~5 min. Experiments were performed at  $33 \pm 0.5^\circ\text{C}$  in a continuous flow of sEBSS solution. A 10 minute wash period was allowed before imaging commenced. After recording resting  $[Ca^{2+}]_i$  levels for 3-5 min, cells were stimulated with P4 (3.6  $\mu$ M). Images were acquired at 0.33 Hz using a 40x oil objective with a CoolSNAP MYO CCD camera controlled by Metsoftware (Molecular Devices, USA). Fluorescence was excited at 488 nm and recorded at 520 nm. Illumination and camera gain setting were maintained constant and fluorescence intensity values are therefore directly comparable between all recordings. A region of interest was drawn round the head and neck region of each cell and several areas were also chosen to assess background fluorescence. Those cells where fluorescence levels fell noticeably during the pre-stimulation period (loss of dye indicating that the cell was dead or dying) were excluded from the analysis. After background correction, resting fluorescence intensity (mean of 25-30 consecutive images collected prior to P4 stimulation) and peak fluorescence intensity (mean of 4-5 consecutive images spanning the peak of the P4-induced  $[Ca^{2+}]_i$  transient) were determined for each cell. P4-induced fluorescence increment for each cell was then calculated by subtracting control fluorescence from peak fluorescence (Supplementary Information Figure S2). Normalisation of background-

corrected fluorescence data was as described previously (Alasmari *et al.*, 2013ab) using  $\Delta F = ((F - F_{rest})/F_{rest}) \times 100\%$ , where  $\Delta F$  is percentage change in intensity,  $F$  is fluorescence intensity at time  $t$ , and  $F_{rest}$  is the mean of 25-30 determinations of  $F$  prior to P4-stimulation. A mean normalised trace was calculated for each experiment by taking the mean  $\Delta F$  of all cells in the experiment ( $\Delta F_{mean}$ ) at each time point. To assess responsiveness to P4 in each cell, the mean and 95% confidence interval of fluorescence intensity were calculated for the period prior to P4 stimulation ( $C \pm c$ ) and the 4-5 images spanning the peak of the transient response ( $T \pm t$ ). The response of that cell was considered significant and classified as a responder if:  $T - t > C + c$  (Kirkman-Brown *et al.*, 2000).

#### **Single cell $[Ca^{2+}]_i$ oscillations**

To assess the occurrence of  $[Ca^{2+}]_i$  oscillations in patients and donors, traces were examined by eye for the occurrence of cyclical increases in  $[Ca^{2+}]_i$ . In 10 patient samples, spontaneous  $[Ca^{2+}]_i$  oscillations were observed during the control period (prior to P4 application) which persisted in the presence of P4. These oscillations often 'masked' the  $[Ca^{2+}]_i$  response to P4 which could not be assessed. These data are presented and discussed separately and are not included in the 3 patient groups.

#### **Fertilisation rate at IVF**

Oocytes were considered normally fertilised when two pronuclei formed (2PN) and two polar bodies were observed. In IVF, the fertilisation rate was calculated from the number of oocytes normally fertilized divided by the total number of inseminated oocytes. Fertilisation rate for IVF was calculated only when four or more mature oocytes (metaphase II) were present.

209

210 **Failed fertilisation**

211 Patients were classified as failed fertilisation (IVF-FF) when no pronuclei were observed  
212 after insemination (minimum of 4 eggs for inclusion of study). Experimentation (CASA, single  
213 cell imaging) was carried out on the day of insemination and therefore the status of the  
214 outcome of IVF treatment was unknown. No ICSI FF patients were included in IVF-FF  
215 analysis.

216

217 **Sperm kinematics**

218 A Hamilton Thorne CEROS computer aided sperm analysis machine was used to measure  
219 sperm sample kinematics and hyperactivation of prepared samples from ART patients  
220 (where sufficient sample was available) and donors (Alasmari *et al.*, 2013a).

221

222 **Experimental solutions**

223 Composition of experimental solutions: HEPES buffered saline, bicarbonate buffered  
224 capacitating medium and sEBBSS are as follows:

225 HEPES buffered saline solution consisted of (in mM):  $\text{CaCl}_2$ , 1.8; KCl, 5.4;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.8;

226 NaCl, 116.4;  $\text{NaH}_2\text{PO}_4$ , 1; D-glucose, 5.5; sodium pyruvate, 2.73; sodium lactate, 41.75;

227 HEPES, 25; BSA, 0.3% (w/v); pH adjusted to 7.4 using NaOH. Bicarbonate buffered

228 capacitating medium consisted of (in mM):  $\text{CaCl}_2$ , 1.8; KCl, 5.4;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.8; NaCl, 116.4;

229  $\text{NaH}_2\text{PO}_4$  1; D-glucose, 5.5; sodium pyruvate, 2.73; sodium lactate, 41.75; sodium

230 bicarbonate, 26; BSA, 0.3% (w/v); pH adjusted to 7.4 using NaOH.

Supplemented Earle's balanced salt solution (sEBSS) contained (in mM); NaH<sub>2</sub>PO<sub>4</sub>, 1.02; KCl, 5.4; MgSO<sub>4</sub>, 0.811; D-glucose, 5.5; Na pyruvate, 2.5; Na lactate, 19.0; CaCl<sub>2</sub>, 1.8; NaHCO<sub>3</sub>, 25.0; NaCl, 118.4 and HEPES, 15 (pH 7.4), supplemented with 0.3% (w/v) BSA.

## **Data analysis**

For analysis, patient samples were divided into three groups IVF(+ve) (successful fertilisation; 62 samples), IVF-FF (failed fertilisation; 8 samples) and ICSI (21 samples, which included 3 samples from patients who had previously failed to fertilise at IVF). Data were analysed using Microsoft Excel™ or GraphPad Prism™ (version 5, GraphPad Software Inc.). Data were assessed for normality using the Shapiro-Wilk test. Statistical significance was determined using Student's t-test, Chi-Square, Kruskal-Wallis test or analysis of variance (ANOVA) as appropriate. Regression analyses of fluorescence increment:resting fluorescence were carried out in Excel using the 'set intercept=zero' option. Regression coefficients were compared as described by Clogg et al (1995) and corrected *post hoc* for multiple comparisons (Gaetano, 2013). Percentage data were converted using the arcsine square root transformation (Sokal and Rohlf, 1981) before statistical analysis to allow application of parametric tests. Holm-Bonferroni correction (Gaetano, 2013) *post hoc* correction was applied as appropriate. Data are presented as mean ± SEM with  $P < 0.05$  indicative of statistical significance.

## Results

### Resting $[Ca^{2+}]_i$ in donor and patient cells

Mean resting  $[Ca^{2+}]_i$  levels (fluo4 fluorescence after background correction) were similar in donors, IVF+ (successful fertilisation) and ICSI patients, but in the eight IVF-FF (failed fertilisation) patients mean resting fluorescence was more than double that in donor cells (fig 1a). Examination of variation within the four categories showed that the majority of donor samples clustered in the range 25-200 and just 1/21 samples (4.8%) exceeded 250. In IVF(+ve) and ICSI populations the proportion of samples with a mean resting fluorescence >250 was similar (4.8% ) but 50% (4/8) of IVF-FF samples exceeded this value ( $P=0.004$ ;  $P=0.002$  and  $P=0.004$  compared to donor, IVF(+ve) and ICSI samples respectively; chi-square; fig 1a).

### $[Ca^{2+}]_i$ responses to P4

As described previously (Kirkman-Brown *et al.*, 2000; Williams *et al.*, 2015), stimulation of human sperm with 3.6  $\mu$ M P4 induced a biphasic  $[Ca^{2+}]_i$  signal composed of an initial transient followed by a sustained  $[Ca^{2+}]_i$  plateau (fig 1b). Initially we analysed the data by normalising fluorescence of fluo4 to the pre-stimulus (resting) level and calculating a mean normalised response for each experiment ( $\Delta F_{mean}$ ; see methods). Using this approach the amplitudes of  $[Ca^{2+}]_i$  transients in samples from ICSI patients and IVF-FF patients were significantly lower than those of donors (fig 1b insert). However, since high levels of resting fluorescence were observed in a large proportion of IVF-FF samples (see above), this approach is potentially misleading since, at high resting  $[Ca^{2+}]_i$ , an equivalent P4-induced  $[Ca^{2+}]_i$  increment will result in a smaller normalised response and also  $[Ca^{2+}]_i$  may approach levels at which dye saturation occurs. To investigate this we examined the relationship

between resting fluorescence and the P4-induced fluorescence increment. Plotting of mean transient amplitude (increment in fluorescence intensity) against mean resting  $[Ca^{2+}]_i$  (resting fluorescence) for each of the 21 donor recordings gave an approximately linear relationship ( $y=2.00x$ ;  $R^2=0.6$ ; fig 1c) over a range of resting fluorescence from 25 to >300. Plotting of equivalent data for the 62 IVF(+ve) samples gave a more complex plot. Most points fell on a straight line very similar to that for donor samples (fig 1d), but in a number of samples ( $\approx 10\%$ ) the mean fluorescence increment fell below the 'expected' range (fig 1d). Similar analysis of the IVF-FF and ICSI patients also showed variation between samples in responsiveness to P4 (fig 1e, f). Overlaying these plots with the data for donor experiments clearly showed that, for a given mean resting fluorescence the mean P4-induced  $[Ca^{2+}]_i$  transient in some ICSI samples and most IVF-FF samples was smaller (fig 1e, f).

To assess the variation of single cell responses to P4,  $[Ca^{2+}]_i$  transient amplitude was assessed in each cell. In donor samples almost all cells ( $98.1 \pm 0.5\%$ ) generated a significant increase in fluorescence upon stimulation with P4 (fig 2a). The great majority of cells in patient samples were also responsive but the proportion was significantly lower in all three groups, particularly in the IVF-FF ( $72.5 \pm 7.7\%$ ;  $P < 0.00005$ ; fig 2a). Plotting of transient amplitude (increment in fluorescence intensity) against resting  $[Ca^{2+}]_i$  (resting fluorescence) for each of the 749 donor cells (21 samples) gave a straight line relationship with a gradient of approximately two ( $y=1.97x$ ;  $R^2=0.52$ ), similarly to that obtained when plotting of mean data for each experiment (compare fig 1c and fig 2b). Overlay of single cell data from IVF(+ve) patient samples showed that whereas most samples followed the distribution seen with donor cells (e.g. fig 2c; Supplementary Information Figure S3a, b), in samples where the mean response deviated from the distribution of donor samples (fig 1d) single cell

301 responses clearly diverged from the distribution of donor cells, even when resting  
 302 fluorescence was well within the 'normal' range (fig 2d; Supplementary Information Figure  
 303 S3c, d). Fitting of linear regressions to single cell distributions confirmed that that these  
 304 differences were significant (fig 2, Supplementary Information Figure S3). Single cell  
 305 analysis and fitting of linear regressions to single cell distributions of ICSI and (more  
 306 particularly) IVF-FF cells showed considerable variability between samples, consistent with  
 307 the scatter of mean values shown in fig 1e, f. In the samples from IVF-FF patients 2310 and  
 308 2236, most cells, including those with the lowest resting fluorescence, deviated strongly  
 309 from the donor distribution resulting in a significantly different regression coefficient (fig 2f,  
 310 Supplementary Information Figure S4b;  $P < 0.00001$ ). In contrast, several of the other IVF-FF  
 311 samples had distributions much closer to that for donor cells (Supplementary Information  
 312 Figure S4a, c, d) and the distribution for patient 2311 was indistinguishable ( $P \approx 1.0$  compared  
 313 to donor cells; fig 2e). Single cell distributions for ICSI samples showed similar variability  
 314 (Supplementary Information Figure S5). The 21 ICSI samples included 3 that were from men  
 315 who had previously failed to fertilise any oocytes at IVF (highlighted red in panel 1f). In one  
 316 of these patients (2714)  $[Ca^{2+}]_i$  responses to P4 deviated markedly from the distribution for  
 317 donor cells (Supplementary Information Figure S5e), but the other two samples (2508,  
 318 2530) fell close to the donor distribution (fig 1f, Supplementary Information Figure S5f).  
 319 Overall, examination of single cell plots of poorly responsive samples from all three patient  
 320 groups indicated that the small P4-induced increment was a genuine characteristic of the  
 321 population and was not specifically associated with high levels of resting fluorescence (high  
 322 resting  $[Ca^{2+}]_i$ ).  
 323 Since IVF patient samples varied considerably in their sensitivity to P4, we investigated the  
 324 relationship between the regression coefficient (P4-induced fluorescence increment:resting



fluorescence) for each sample and fertilisation rate of that sample at IVF. There was a significant positive relationship between these variables ( $P=0.0004$ ;  $R^2=0.14$ ; fig 3a). Furthermore, separation of IVF samples into those with a regression coefficient  $<1.0$  (increment in fluorescence less than resting fluorescence) and those with a coefficient of  $\geq 1.0$  gave mean fertilisation rates of  $31.0 \pm 7.6\%$  ( $n=55$ ) and  $61.8 \pm 3.8\%$  ( $n=15$ ) respectively ( $P=0.0015$ ).

#### Occurrence of P4-induced $[Ca^{2+}]_i$ oscillations

Single cell imaging allows the detection of complex  $[Ca^{2+}]_i$  signals that are masked in populations measurements. A common observation is the occurrence of  $[Ca^{2+}]_i$  oscillations, superimposed on the plateau phase of the P4-induced  $[Ca^{2+}]_i$  response (fig 4a). Figure 4b shows the proportion of cells in which P4 induced  $[Ca^{2+}]_i$  oscillations occurred. In all three patient groups we observed induction of  $[Ca^{2+}]_i$  oscillations upon stimulation with P4 but whereas frequency of occurrence in IVF(+ve) and ICSI samples was 20-25%, similar to donor controls ( $21.4 \pm 5.0\%$ ,  $n=22$ ; fig 4b), in IVF-FF samples the proportion of oscillating cells was only  $11.2 \pm 6.7\%$  ( $n=8$ ). Variation between the eight IVF-FF patients was considerable (proportion of oscillating cells ranged from 0-54%), but the proportion of samples in which no cells generated  $[Ca^{2+}]_i$  oscillations (3/8) significantly exceeded that in donors (1/21;  $P<0.02$ ) or IVF(+ve) samples (2/62;  $P<0.0005$ ). Plotting of the relationship between generation of  $[Ca^{2+}]_i$  oscillations (% cells oscillating) and fertilisation for all IVF samples (IVF(+ve) and IVF-FF) revealed a weak but significant correlation ( $P=0.02$ ;  $R^2=0.054$ ; fig 3b). In all patient groups the period of P4-induced oscillations was slightly shorter than in controls, but this difference was significant only in the IVF-FF group, where oscillation

period was  $44.3 \pm 2.6$  s ( $n=48$  cells) compared to  $54.8 \pm 1.3$  s ( $n=183$  cells) in donors (fig 4c;  $P < 0.05$ ).

### Spontaneous calcium oscillations

In  $\approx 8\%$  of donor cells (63/749) we observed spontaneous  $[Ca^{2+}]_i$  oscillations, as described previously (Sanchez-Cardenas *et al.*, 2014) but amplitudes were small compared to those induced by P4 ( $\Delta\text{fluorescence} = 31 \pm 3.5\%$  and  $113 \pm 26\%$  respectively;  $P < 0.001$ ). However, in 10 patient samples, all of which fertilised at IVF (fertilisation rate =  $60.7 \pm 7.4\%$ ), we observed large spontaneous  $[Ca^{2+}]_i$  oscillations similar in amplitude to those induced by P4 (fig 4d). These patients were not included in analysis of P4-induced  $[Ca^{2+}]_i$  signalling because spontaneous activity masked/distorted the response to P4 (fig 4d). Stimulation with P4 caused an increase in baseline  $[Ca^{2+}]_i$  but spontaneous oscillations persisted and no clear P4-induced transient could be discerned (figure 4d). Neither the amplitude nor the frequency of these spontaneous  $[Ca^{2+}]_i$  oscillations was significantly altered in the presence of P4 ( $P > 0.05$ ). Examination of the relationship between the proportion of spontaneously oscillating cells in each of these 10 patients and fertilisation rate at IVF showed a weak, non-significant relationship ( $p = 0.19$ ; Supplementary Information Figure S6).

### **Motility of patient and donor sperm**

All donor and IVF patient samples included in this study were assessed by CASA prior to experimentation. Due to the volume and cell concentration of most ICSI samples, accurate CASA analysis was not possible. Analysis of motility data (total and progressive) showed no significant differences between donor and patient populations, but motility kinematics were clearly altered in patient samples. Figure 5 shows the distributions of amplitude of lateral

head movement (ALH) (panel a), curvilinear velocity (VCL) (panel b), linearity (panel c) and percentage of hyperactivated cells (panel d) for the donor, IVF(+ve) and IVF-FF groups. Patient samples had higher linearity and lower ALH and VCL (IVF-FF only). Consistent with these differences, both IVF(+ve) ( $10.2 \pm 0.9\%$ ,  $n=62$ ) and IVF-FF ( $3.1 \pm 1.1\%$ ,  $n=8$ ) had a significantly lower percentage of hyperactivated cells when compared to donor samples ( $18.0 \pm 2.3\%$ ,  $n=21$ ;  $P=0.00005$  and  $0.0007$  respectively). The percentage of hyperactivated cells in IVF-FF samples was also significantly lower than in the IVF(+ve) group,  $P=0.02$ .

## Discussion

CatSper channels are the main source of  $\text{Ca}^{2+}$  entry in human sperm (Brenker *et al.*, 2012), and studies in which CatSper activity and fertility (outcome of IVF treatment) of sperm populations have been assessed suggest that even minor abnormalities of CatSper function may affect fertility (Krausz *et al.*, 1995, 1996; Qi *et al.*, 2007; Lishko and Kirichok 2010; Williams *et al.*, 2015). However, assessment of CatSper function in sperm populations masks the occurrence of cell-cell variation within the sample which may be of functional or diagnostic significance. We used single cell imaging to explore the heterogeneity of single cell  $[\text{Ca}^{2+}]_i$  responses to P4 in donor and patient samples and to assess how this relates to fertilising ability (by IVF) of the sperm population. Our data show not only that P4-evoked and spontaneous  $[\text{Ca}^{2+}]_i$  signals vary between cells in a single ejaculate (as has been described previously for cells from 'healthy' donors), but that there is clear variation between and within patient types (as assessed by an ART clinic) in regards to the proportion of cells that respond to the CatSper agonist P4 and the nature of the responses elicited.

Resting and P4-stimulated  $[\text{Ca}^{2+}]_i$  in donor and patient sperm

396 Analysis of resting (pre-stimulus) fluorescence showed wide variation between samples  
 397 both within and between patient and donor groups. In particular, in the IVF-FF patient  
 398 group, half of the samples showed an unusually high resting fluorescence. Though we  
 399 cannot discount the possibility that this reflects abnormalities of dye loading/behaviour in  
 400 these samples, it suggests that high resting sperm  $[Ca^{2+}]_i$  may be characteristic of some sub-  
 401 fertile men. Increased resting  $[Ca^{2+}]_i$  could be due to enhanced tonic  $Ca^{2+}$ -influx through  
 402 CatSper, for instance due to unusually high  $pH_i$  or depolarised  $V_m$  (Brown et al., 2016).  
 403 Alternatively, impairment of  $Ca^{2+}$  clearance mechanisms may cause elevated resting  $[Ca^{2+}]_i$ .  
 404 For instance, sperm from plasma membrane calcium ATPase 4 (PMCA4)-null mice have  
 405 increased  $[Ca^{2+}]_i$ , though the loss of motility in such cells is far more severe than the effects  
 406 observed in this study (Okunade et al., 2004; Schuh et al., 2004).

407

408 Since resting fluorescence varied between donor/patient groups, simple normalisation of  
 409 fluorescence to pre-stimulus levels is potentially misleading. If the high levels of resting  
 410 fluorescence in these samples genuinely reflect high  $[Ca^{2+}]_i$  then (i) a 'normal' P4-induced  
 411 CatSper activation/ $Ca^{2+}$  influx will give a smaller proportional increase in fluorescence and  
 412 (ii) the dye may approach saturation, underestimating the  $[Ca^{2+}]_i$  signal. Therefore, to  
 413 analyse the amplitude of  $[Ca^{2+}]_i$  responses to progesterone we investigated the relationship  
 414 between resting fluorescence and the P4-induced fluorescence increment. Plotting the data  
 415 from donor samples either using sample means or individual cells gave a clear, linear  
 416 relationship that showed no evidence of dye saturation over the range of resting  
 417 fluorescence observed. For most patient samples a similar relationship between resting  
 418 fluorescence and the P4-induced fluorescence increment was seen but in approximately

10% of IVF(+ve) patients and one third of IVF-FF and ICSI patients the response to P4 fell clearly below the 'normal' range.

Examination of the single cell resting fluorescence:P4-induced increment plots from samples which gave 'sub-normal' responses to P4 suggests that the nature of the underlying lesion varies. In each of the patient groups we observed some samples that generated clearly linear scattergrams but responses to P4 were smaller than those obtained with donor sperm, such that the gradient of the plot was significantly lower. Such reduced sensitivity could occur due to poor expression of CatSper channels (Tamburrino et al.; 2015). Alternatively, expression of a mutant CatSper channel with reduced conductance, as has recently been described for mouse sperm lacking CatSper $\zeta$  (Chung et al, 2017), could produce this phenotype. A second pattern seen in patients samples was a 'cloud' of points to the right of/below the donor distribution. Resting fluorescence was unusually high in some of these samples, but it is also notable that the ratio of P4-induced increment to resting fluorescence varied greatly between cells, indicating great intra-sample variation in resting  $[Ca^{2+}]_i$  and/or expression of functional CatSper. Data from patient 2236 produced an intriguing 'hybrid' plot including cells that responded 'normally' to P4 and cells that gave a negligible/zero response, suggesting that only a sub-population of these sperm express functional CatSper. Significantly, though the response to P4 was impaired in a significant proportion of the 91 patients where analysis was possible, we did not detect any men who were null or 'functionally null' (Williams et al., 2015) for CatSper in every cell, indicating that such patients are very rare.

P4-induced  $[Ca^{2+}]_i$  signalling and fertility

To assess the functional significance of this variability in response to P4, we examined the relationship between P4-sensitivity (regression coefficient of the single cell scatter plot) and fertilisation rate of the sample in IVF. Consistent with previous studies on P4-induced population  $[Ca^{2+}]_i$  signals (Krausz *et al.*, 1995, 1996; Alasmari, *et al.*, 2013a; Williams *et al.*, 2015), the data showed a significant positive relationship. Taken together with our observation that most IVF patients had a high proportion of cells in which a significant response to P4 was detected (mean~95%), this suggests the existence of a threshold level of single sperm CatSper activity/P4 sensitivity below which fertilisation competence of the cell is compromised. Notably, some IVF-FF samples responded 'normally' or near-normally to P4 - failure of such samples to fertilise probably reflects lesions not associated with  $[Ca^{2+}]_i$  signalling.

#### $[Ca^{2+}]_i$ oscillations in donors and patient sperm.

Upon stimulation of human sperm with P4, the initial  $[Ca^{2+}]_i$  transient is followed, in a subset of cells, by  $[Ca^{2+}]_i$  oscillations which are dependent on influx of extracellular  $Ca^{2+}$  but appear also to involve repetitive mobilisation of  $Ca^{2+}$  stores (Harper *et al.*, 2004; Kirkman-Brown *et al.*, 2004; Bedu-Addo *et al.*, 2007; Sanchez-Cardenas *et al.*, 2014; Mata-Martinez *et al.*, 2018). These oscillations are reported both to regulate activity of the flagellum, potentially modifying sperm behaviour to facilitate penetration of the oocyte vestments (Harper *et al.*, 2004), and to be associated with low levels of acrosome reaction (Harper *et al.*, 2004; Sanchez-Cardenas *et al.*, 2014). In this study P4-induced  $[Ca^{2+}]_i$  oscillations were observed in cells of donors and all patient groups. However, in the failed fertilisation (IVF-FF) group the mean percentage of cells that generated oscillations upon P4 treatment was only half that in donors and in the IVF(+ve) group and the proportion of samples that failed

totally to generate oscillations was significantly higher in the IVF-FF group.  $[Ca^{2+}]_i$  responses to P4 were small in these samples, consistent with the dependence of oscillations on background  $Ca^{2+}$  influx through CatSper. However, in the large IVF(+ve) group (n=62) generation of oscillations showed no significant relationship to P4-induced fluorescence increment ( $P=0.55$ ;  $R^2=0.006$ ) or to the regression coefficient of the single cell (fluorescence increment:resting fluorescence) scatter plot ( $P=0.09$ ;  $R^2=0.05$ ), suggesting that other aspects of  $Ca^{2+}$ -handling, presumably including activity of the  $Ca^{2+}$ -store, are also important and may lead to failure of oscillations and reduced fertility.

Samples from 10 IVF patients included sperm that showed large spontaneous  $[Ca^{2+}]_i$  oscillations that persisted in the presence of P4 with no significant change in amplitude or frequency and largely masked the P4-induced  $[Ca^{2+}]_i$  transient. The occurrence of spontaneous oscillations might indicate attainment of an advanced level of capacitation (Baldi *et al.*, 1991; Mendoza and Tesarik 1993; Garcia and Meizel 1999; Kirkman-Brown *et al.*, 2000). If this is correct, the variation in their occurrence reflects innate differences between samples since all IVF patient samples were prepared and their responses assessed in the same way. Sanchez-Cardenas *et al.* reported recently that 98% of cells generating spontaneous  $[Ca^{2+}]_i$  oscillations fail to undergo acrosome reaction upon stimulation with P4, and concluded that this spontaneous activity may suppress premature occurrence of acrosome reaction, though mechanisms are still unknown (Sanchez-Cardenas *et al.*, 2014). All patients in which these large, spontaneous  $[Ca^{2+}]_i$  oscillations were observed successfully fertilised at IVF.

Impaired  $[Ca^{2+}]_i$  signalling and sperm function

491 P4-induced (CatSper-mediated)  $\text{Ca}^{2+}$  influx and P4-induced  $[\text{Ca}^{2+}]_i$  oscillation were  
492 statistically associated with poor fertilisation at IVF. Both these aspects of  $\text{Ca}^{2+}$  signalling  
493 have been implicated in regulation of human sperm motility. Analysis of CASA recordings  
494 from the samples used in this study showed significant differences in kinematics between  
495 donor cells and the IVF-FF samples. These findings strongly support previous reports of  
496 reduced hyperactivation in subfertile patients (Alasmari *et al.*, 2013a) and suggest that the  
497 relationship between impaired P4 activation of CatSper, abnormal  $[\text{Ca}^{2+}]_i$  signalling, and  
498 poor IVF success rate reported here (and in previous studies on population responses;  
499 Krausz *et al.*, 1995, 1996; Alasmari, *et al.*, 2013a) reflects, at least in part, the effect of  
500 compromised  $[\text{Ca}^{2+}]_i$  signalling on regulation of sperm motility (Alasmari *et al.*, 2013b).  
501 However, impaired  $[\text{Ca}^{2+}]_i$  signalling is also likely also to affect capacitation, regulation of  
502 acrosome reaction and viability. We have observed striking differences between patient  
503 samples in resting  $[\text{Ca}^{2+}]_i$ , single-cell P4-sensitivity and generation of  $[\text{Ca}^{2+}]_i$  oscillations;  
504 future studies should consider the relative incidence, underlying causes and functional  
505 significance of these abnormalities for human male fertility.  
506



507

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514

**515 Author's roles**

516 MCK and SGB performed the single cell imaging. MCK, SJP, SJC and SGB analysed the data.  
517 MR and SMDS were involved in recruiting patients and seeking informed consent. E.D.  
518 processed the patient samples. SJP, SMDS and CLRB designed the study. SJP, SGB and CLRB  
519 obtained funding for the study. All authors contributed to the construction, writing, analysis  
520 and approval of the final manuscript.

521

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528

**529 Conflict of interest**

- 530 C.L.R.B is the editor-in-chief of Molecular Human Reproduction and Chair of the World  
531 Health Organisation Expert Synthesis Group on Diagnosis of Male infertility (2012-2017).  
532 C.L.R.B. receives lecturing fees from Merck and Ferring.  
533  
534  
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## Figure Legends

Figure 1. Resting fluorescence and population responses to P4.

Panel a: Mean resting fluorescence for donor (black; n=21 samples), IVF(+ve) (blue; n=62 samples), IVF-FF (red; n=8 samples) and ICSI (green; n=21 samples) sample. Plots show individual values and mean  $\pm$  SEM.

Panel b:  $[Ca^{2+}]_i$  responses to P4 in donors (black), IVF(+ve) (blue), IVF-FF (red) and ICSI (green) groups. Arrow shows time of progesterone addition. Plots were obtained by normalizing data to pre-stimulus level, calculating the population response (mean of all cells imaged -  $\Delta F_{mean}$ ) for each sample and then averaging these for the donors (n=21 experiments) and for each of the 3 patient groups: IVF(+ve) (n=62 experiments), IVF-FF (n=8 experiments) and ICSI (n=21 experiments). Inset shows mean ( $\pm$  SEM) normalized transient amplitude for each data set. Asterisks indicate  $P < 0.05$  (\*) and  $P < 0.01$  (\*\*) with respect to donor samples.

Panel c: Relationship between mean resting fluorescence and mean fluorescence increment for 21 donor samples. Line shows fitted regression ( $y = 2.0x$ ;  $R^2 = 0.6$ ).

Panels d-f: Relationship between mean resting fluorescence and mean fluorescence increment for IVF(+ve) ((d) blue, n=62 samples); IVF-FF ((e) red; n=8 samples) and ICSI ((f) green, n=21 samples) respectively. Numbered points (highlighted yellow in panel (d) for clarity) show patients for whom single cell analysis is shown in fig 2 and Supplementary Information Figures S3-5. Points highlighted in red in panel f (ICSI) are patients who had previously failed to fertilise any oocytes at IVF. In each of panels d-f black points and fitted regression show data from donor samples for comparison.

Figure 2. Single cell P4-induced  $[Ca^{2+}]_i$  transients.

Panel a: Proportion of cells showing significant increase in fluorescence upon application of 3.6  $\mu$ M P4. Asterisks indicate  $P < 0.005$  (\*\*\*) and  $P < 0.00005$  (\*\*\*\*\*) with respect to donor samples. Panel b: Relationship between resting fluorescence and fluorescence increment for 749 cells from 21 donor samples. Line shows fitted regression ( $y = 1.97x$ ;  $R^2 = 0.52$ ). Panels c-f show examples of relationship between mean resting fluorescence and mean fluorescence increment in two IVF(+ve) patients ((c) and (d); 32 and 37 cells respectively; blue symbols); and two IVF-FF patient ((e) and (f) 52 cells and 53 cells respectively; red symbols). In each of panels c-f black points and show data from donor cells (panel b) for comparison. Numbers in each panel are patient code (for comparison with fig 1) and P values show comparison of patient regression coefficient with that for donor cells.

Figure 3.  $[Ca^{2+}]_i$  signaling and fertilisation rate.

Panel a: P4 sensitivity and IVF fertilisation rate. X-axis is regression coefficient calculated for the relationship between single cell resting fluorescence and P4-induced fluorescence for each sample. Y-axis value is fertilisation rate for each sample. Data from 62 IVF(+ve) and 8 IVF-FF samples.

Panel b: Induction of  $[Ca^{2+}]_i$  oscillations by P4 and IVF fertilization rate. Plot shows relationship between proportion of cells in which oscillations were induced by treatment with P4 (3.6  $\mu$ M) and the fertilization rate (%) achieved at IVF with that sample. Data from 62 IVF(+ve) and 8 IVF-FF samples.

Figure 4. Calcium oscillations in progesterone-stimulated cells.

a: Representative trace of a P4-induced  $[Ca^{2+}]_i$  oscillation in single spermatozoon of an IVF (+ve) patient. 3.6  $\mu$ M P4 was added at the arrow. b: Proportion of cells that generated  $[Ca^{2+}]_i$  oscillations when stimulated with 3.6  $\mu$ M P4. Bars show mean  $\pm$  SEM for donors (black, n=21 experiments), IVF(+ve) (blue, n=62 experiments), and IVF-FF (red, n=8 experiments and ICSI (green, n=21 experiments).  $P < 0.05$  (\*). c: Mean  $[Ca^{2+}]_i$  oscillation period ( $\pm$ SEM); donors (black, n=143 cells), IVF(+ve) (blue, n=582 cells), IVF-FF (red, n=43 cells;  $P < 0.05$  (\*)) and ICSI (green, n=162 cells) and d: Example of cell (successful IVF patient) generating large spontaneous  $[Ca^{2+}]_i$  oscillations, which persisted during P4 exposure.

Figure 5. Kinematics and hyperactivation of donor and patient cells (assessed by CASA). Plots show mean  $\pm$  SEM and distribution of individual values for: (a) amplitude of lateral head movement (ALH;  $\mu$ m); (b) curvilinear velocity (VCL;  $\mu$ m/s) (c) linearity (%) (d) hyperactivation (%). Asterisks indicate statistical difference from donors except where indicated \* $p < 0.05$ . \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

Supplementary Figure S1. Comparison of calcium signalling in donor sperm that were capacitated in commercially available Quinn's media or laboratory prepared bicarbonate-buffered media. Data are from split (paired) samples. Sperm were isolated using a 40%-80% Percoll discontinuous density gradient procedure in a HEPES-buffered, BSA-free saline. Mean resting fluorescence (a) and P4-induced  $[Ca^{2+}]_i$  increase expressed as fluorescence increment (b) or normalised to pre-stimulus levels (delta F % (c)), were not significantly different between treatment groups. Data are from 137 (Quinn's medium) and 157 (laboratory medium) cells from 4 donors.

Supplementary Figure S2. Calculation of resting fluorescence and fluorescence increment.

After background correction, resting fluorescence intensity (mean of 25-30 consecutive images collected prior to P4 stimulation) and peak fluorescence intensity (mean of 4-5 consecutive images spanning the peak of the P4-induced  $[Ca^{2+}]_i$  transient) were determined for each cell. P4-induced fluorescence increment was then calculated by subtracting control fluorescence from peak fluorescence.

Supplementary Figure S3: Examples of relationship between mean resting fluorescence and mean fluorescence increment in four IVF(+ve) patients (panels a-d; 39, 24, 53 and 30 cells respectively; blue symbols). In each panel the black points show data from donor cells for comparison (749 cells from 21 donor samples). Numbers in each panel are patient code (for comparison with fig 1) and P values show comparison of patient regression coefficient with that for donor cells.

Supplementary Figure S4. Examples of relationship between mean resting fluorescence and mean fluorescence increment in four IVF-FF patients (panels a-d; 34, 13, 33 and 23 cells respectively; red symbols). In each of panel the black points show data from donor cells for comparison (749 cells from 21 donor samples). Numbers in each panel are patient code (for comparison with fig 1) and P values show comparison of patient regression coefficient with that for donor cells.

Supplementary Figure S5. Examples of relationship between mean resting fluorescence and mean fluorescence increment in four ICSI patients (panels a-d; 21, 15, 37 and 29 cells respectively; green symbols). In each panel the black points show data from donor cells for

comparison (749 cells from 21 donor samples). Numbers in each panel are patient code (for comparison with fig 1) and P values show comparison of patient regression coefficient with that for donor cells.

Supplementary Figure S6. Spontaneous  $[Ca^{2+}]_i$  oscillations and IVF fertilization rate.

Plot shows relationship between proportion of cells in which large spontaneous  $[Ca^{2+}]_i$  oscillations were observed (n=10 samples where such oscillations occurred) and the fertilization rate (%) achieved at IVF with that sample.

Supplementary Figure S7. Selection criteria for patient inclusion. 441 couples attended the ACU for ART treatment during the study period. 270 of these (61%) were consented for research (donor sperm for female same sex couples was not included in study). For 189 (70%) of the consented couples there was adequate surplus sample for research and expertise was available on the day to carry out experiments. 143 of these 189 patients (76%) met the minimum 4 egg insemination criteria and of these there were 101 samples (71%) where we successfully obtained  $[Ca^{2+}]_i$  imaging data.

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761

Figure 1

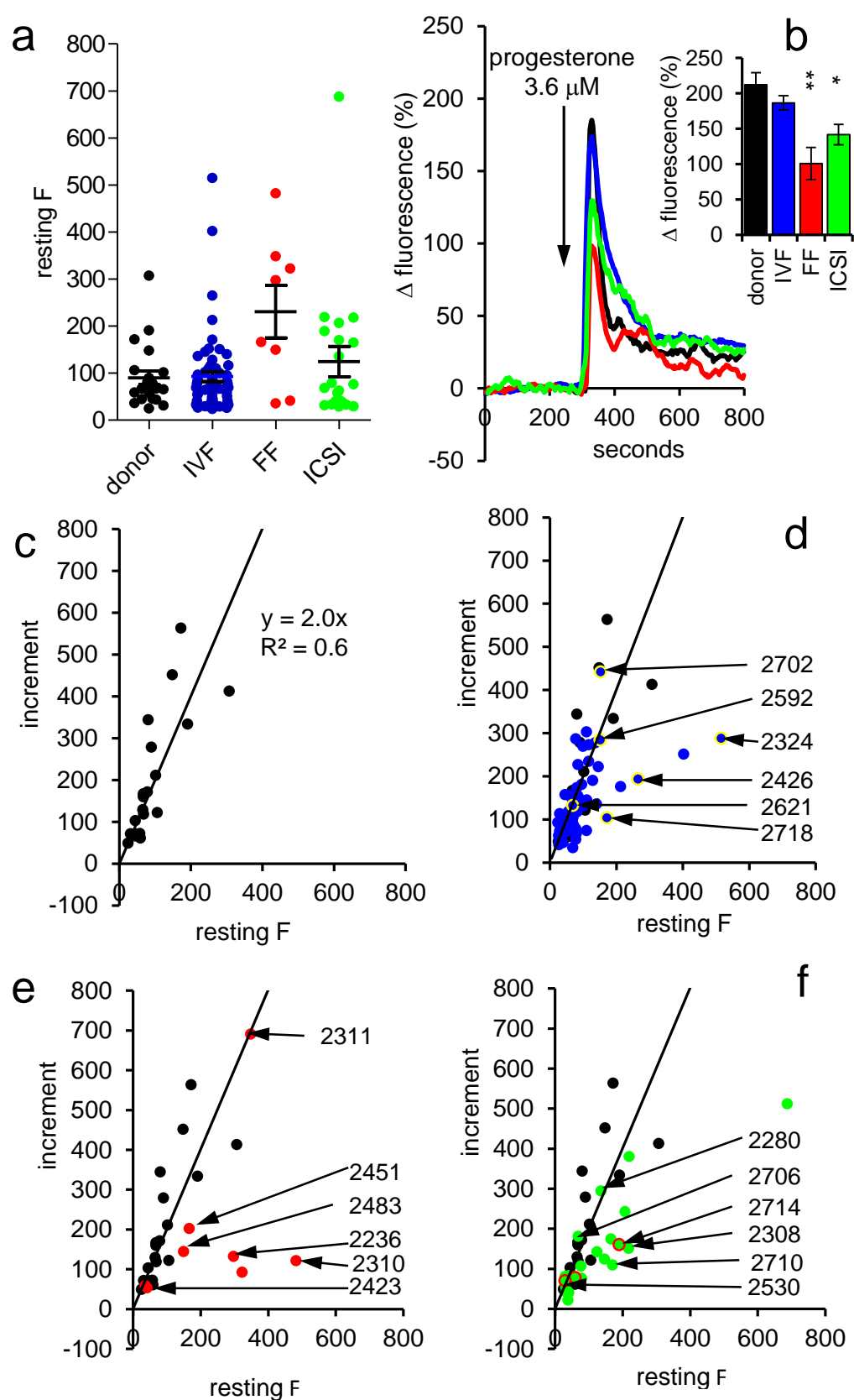
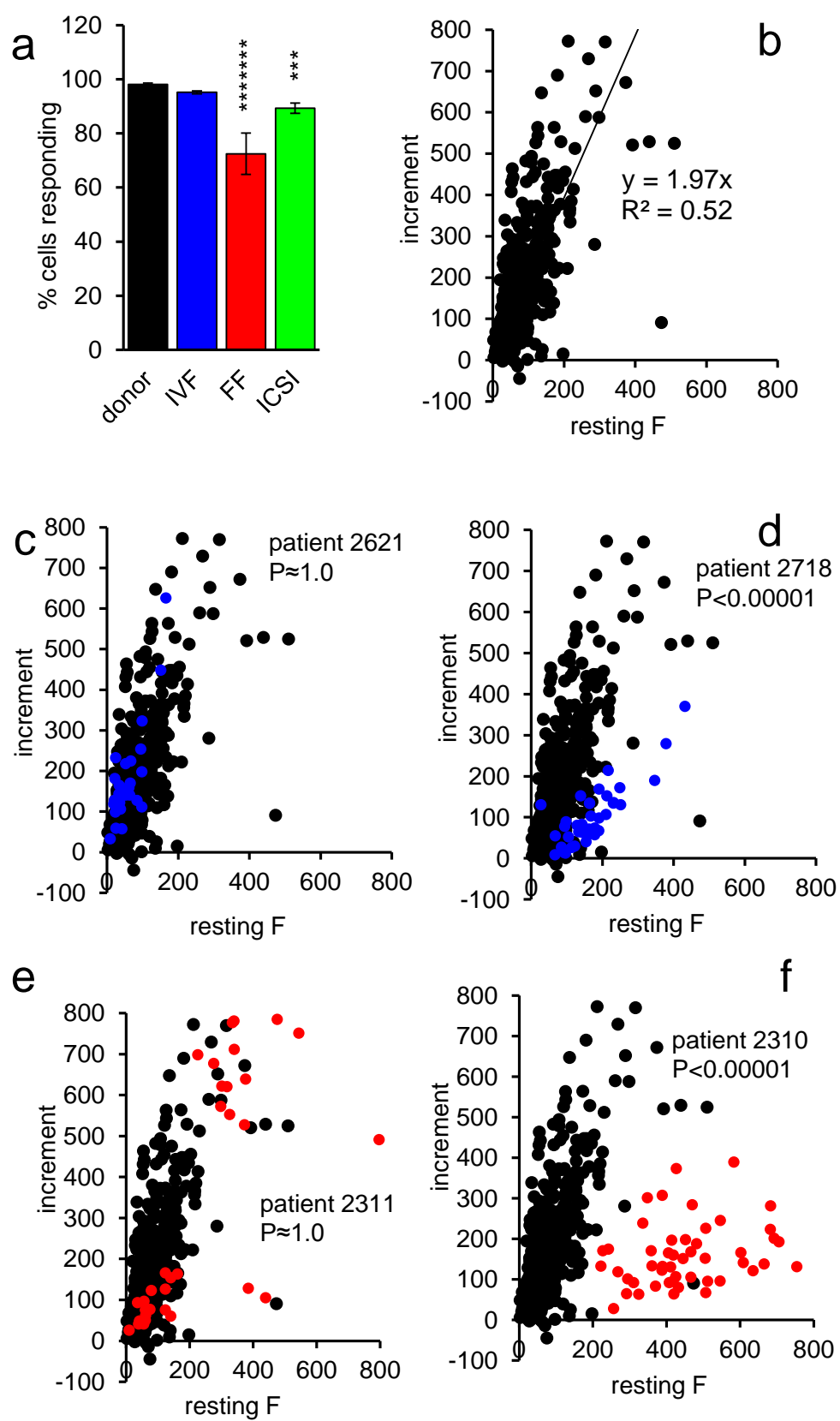
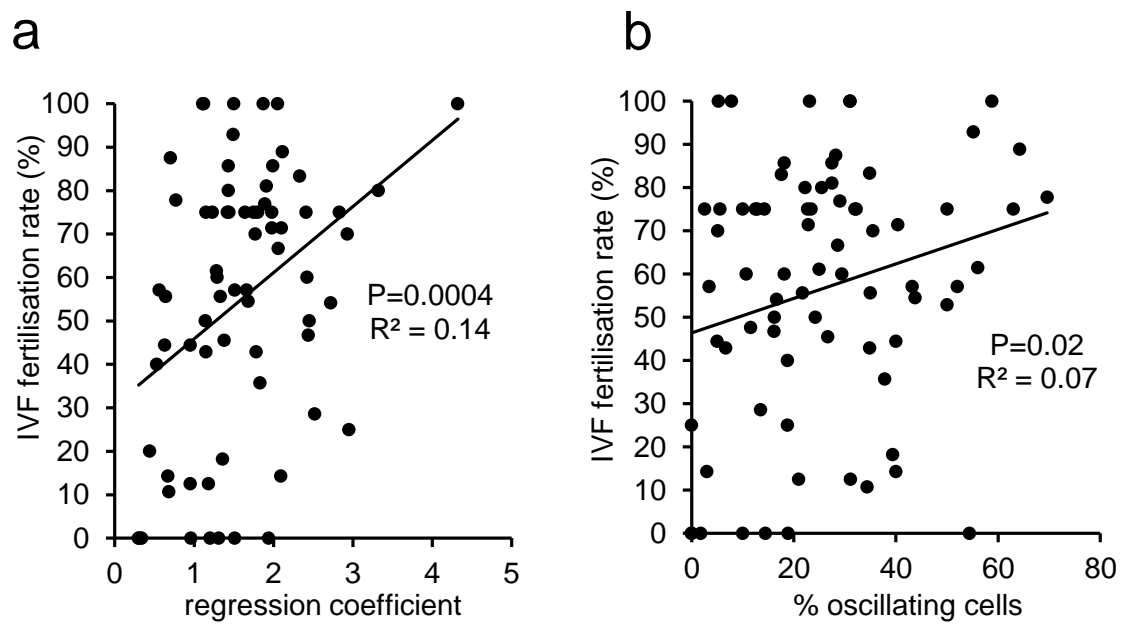


Figure 2



764

Figure 3



765

Figure 4

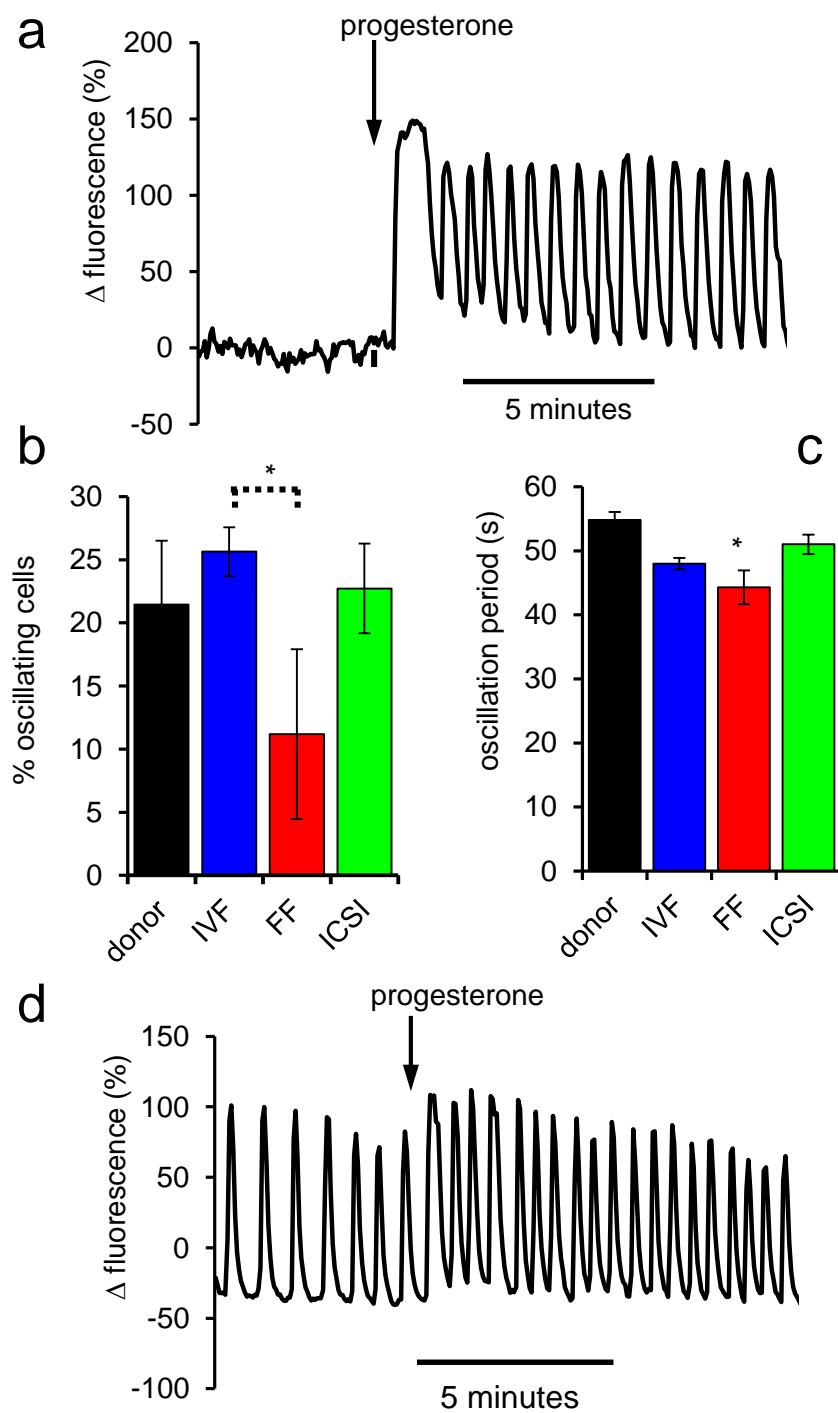
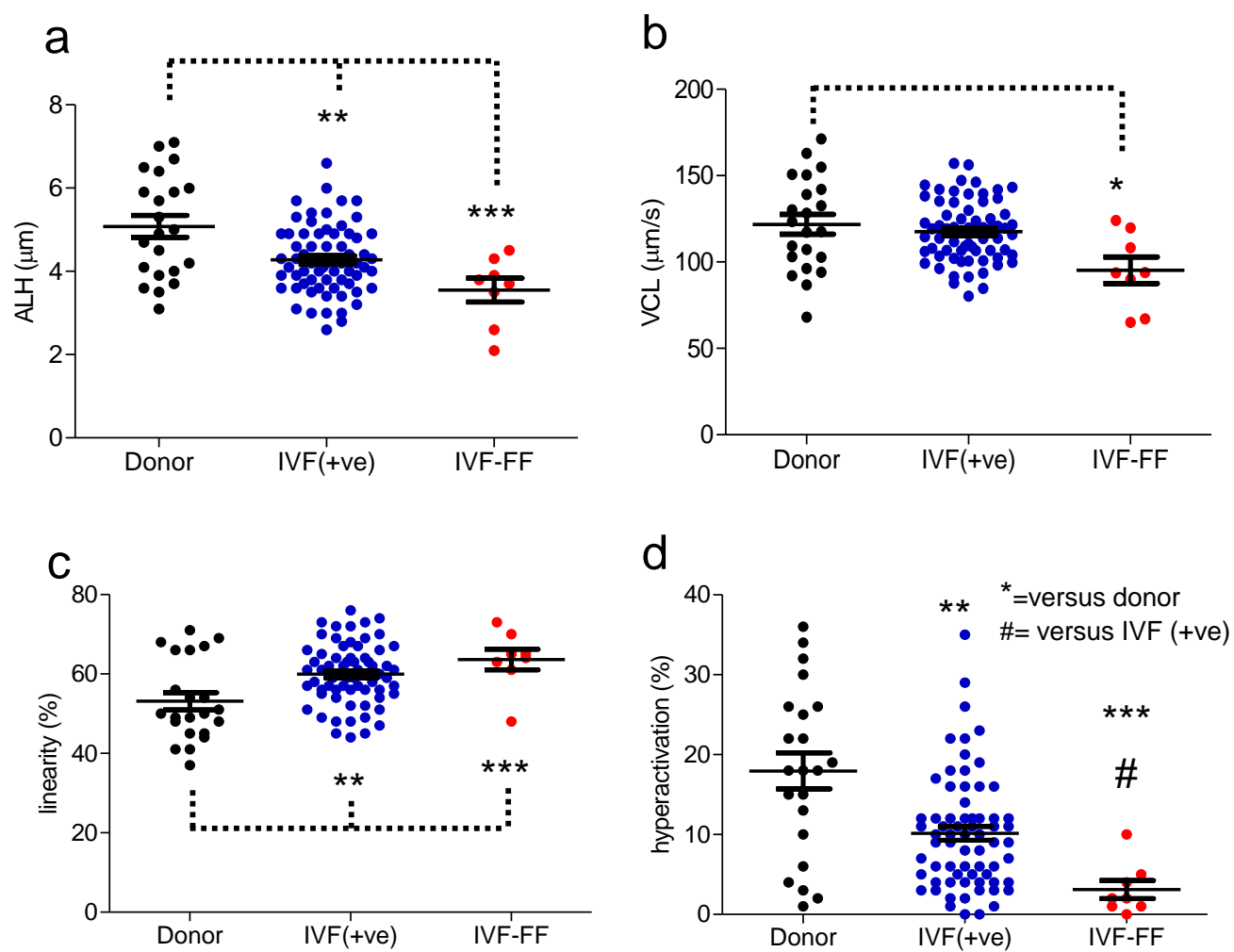
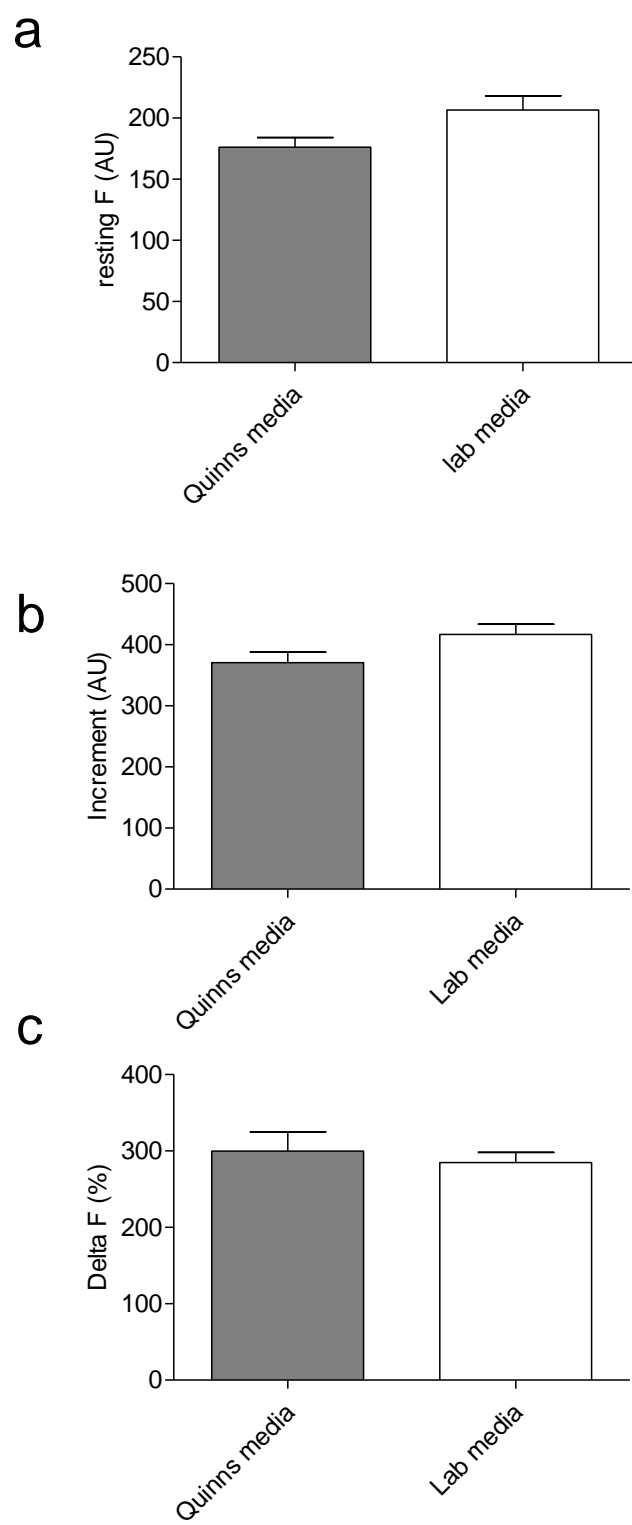




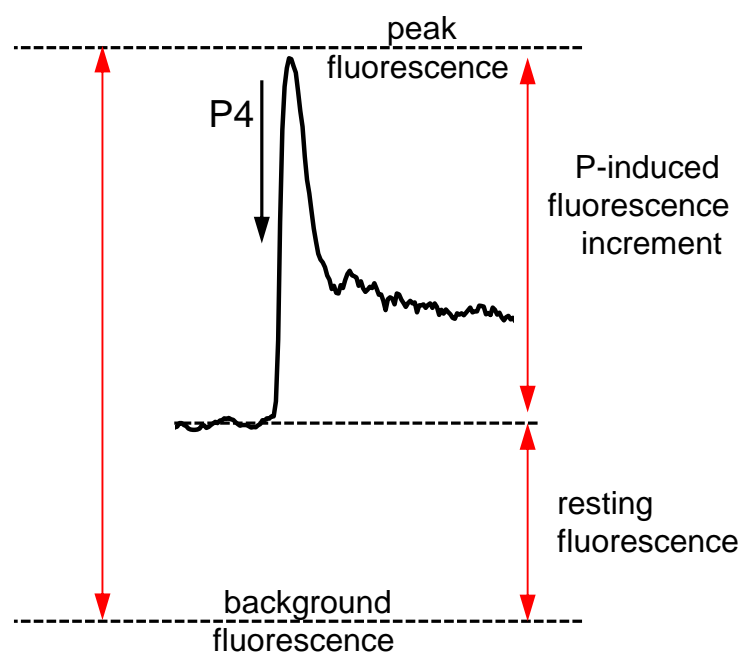
Figure 5



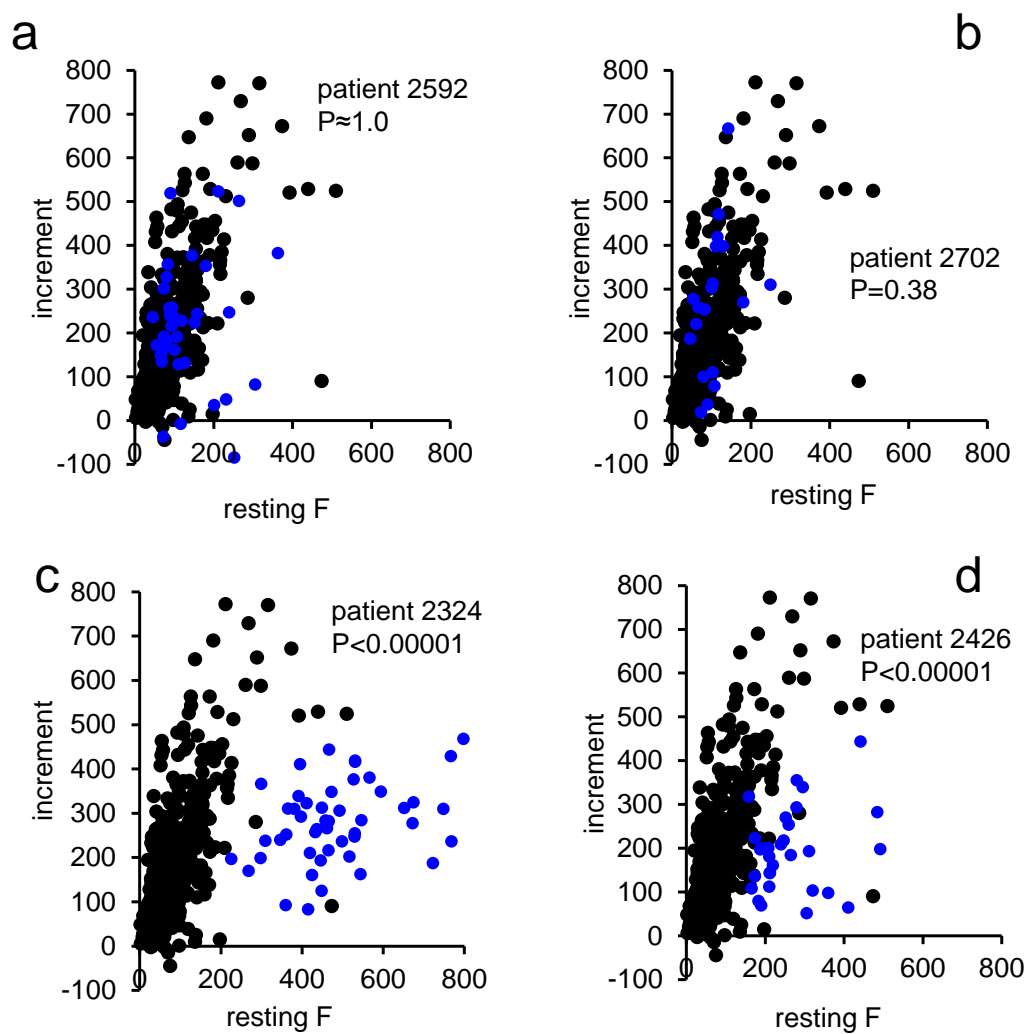
## Suppl fig 1



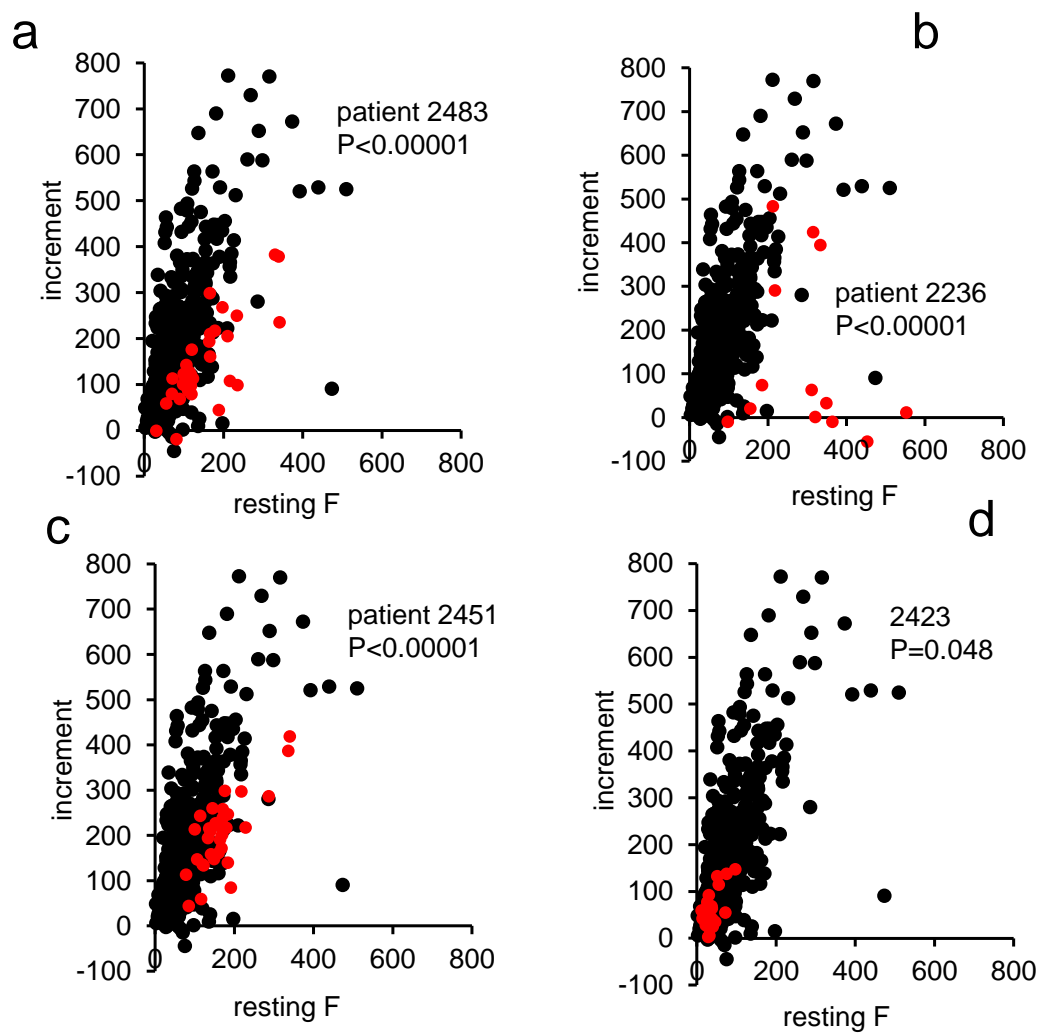
## Suppl fig 2



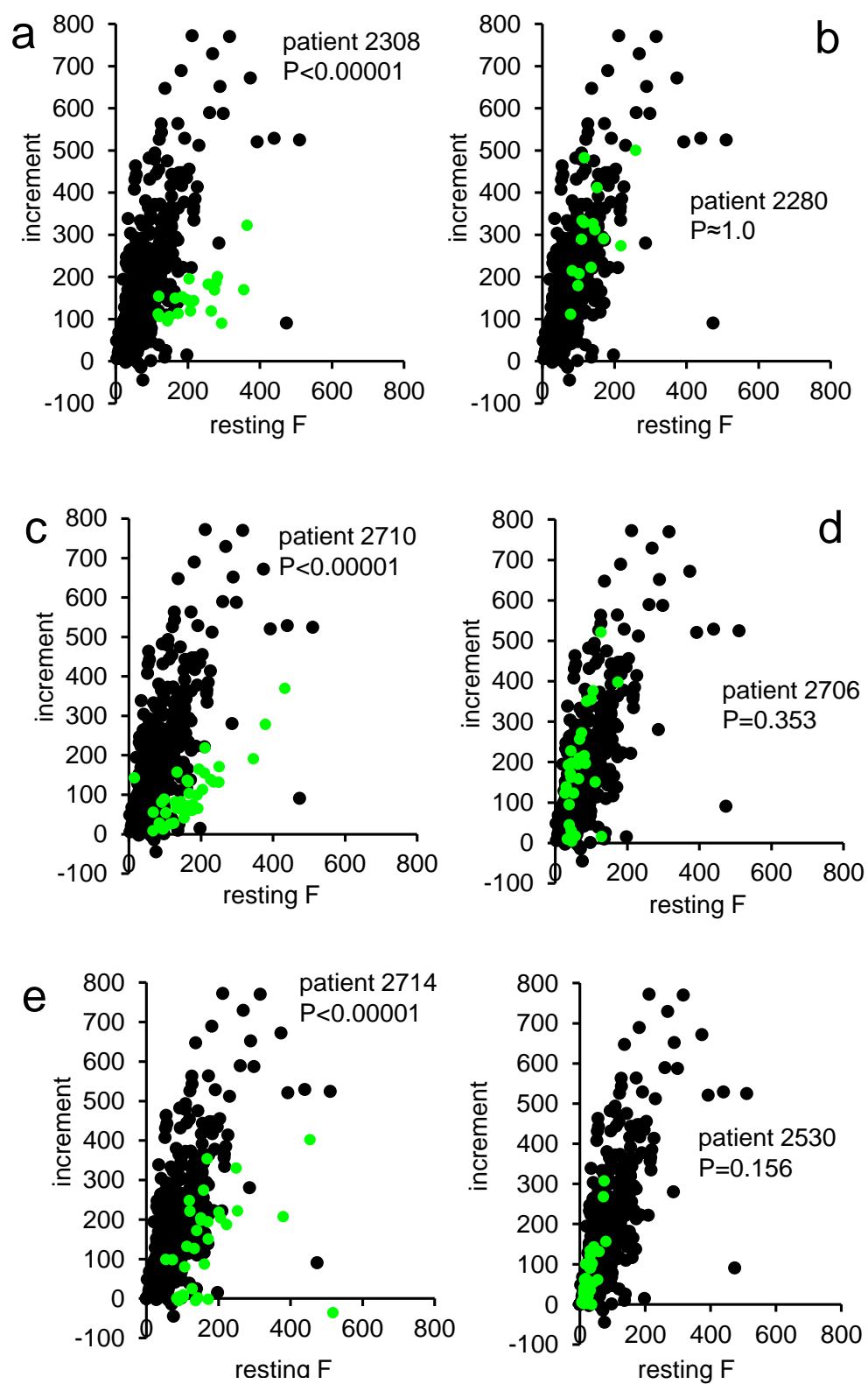
## Suppl fig 3



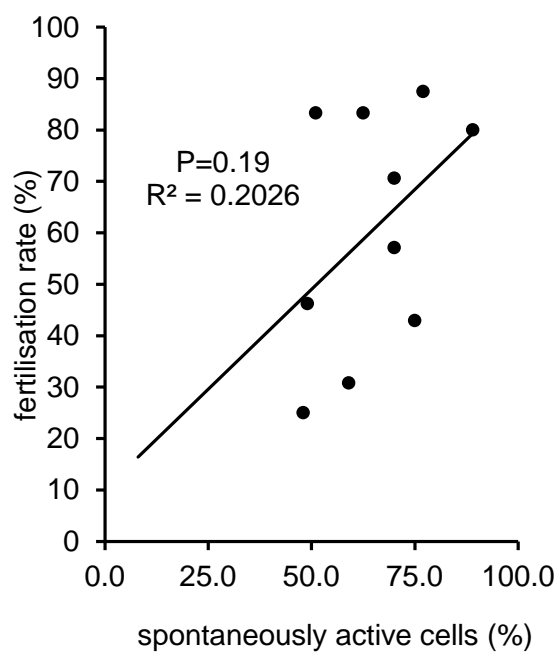
Suppl fig 4



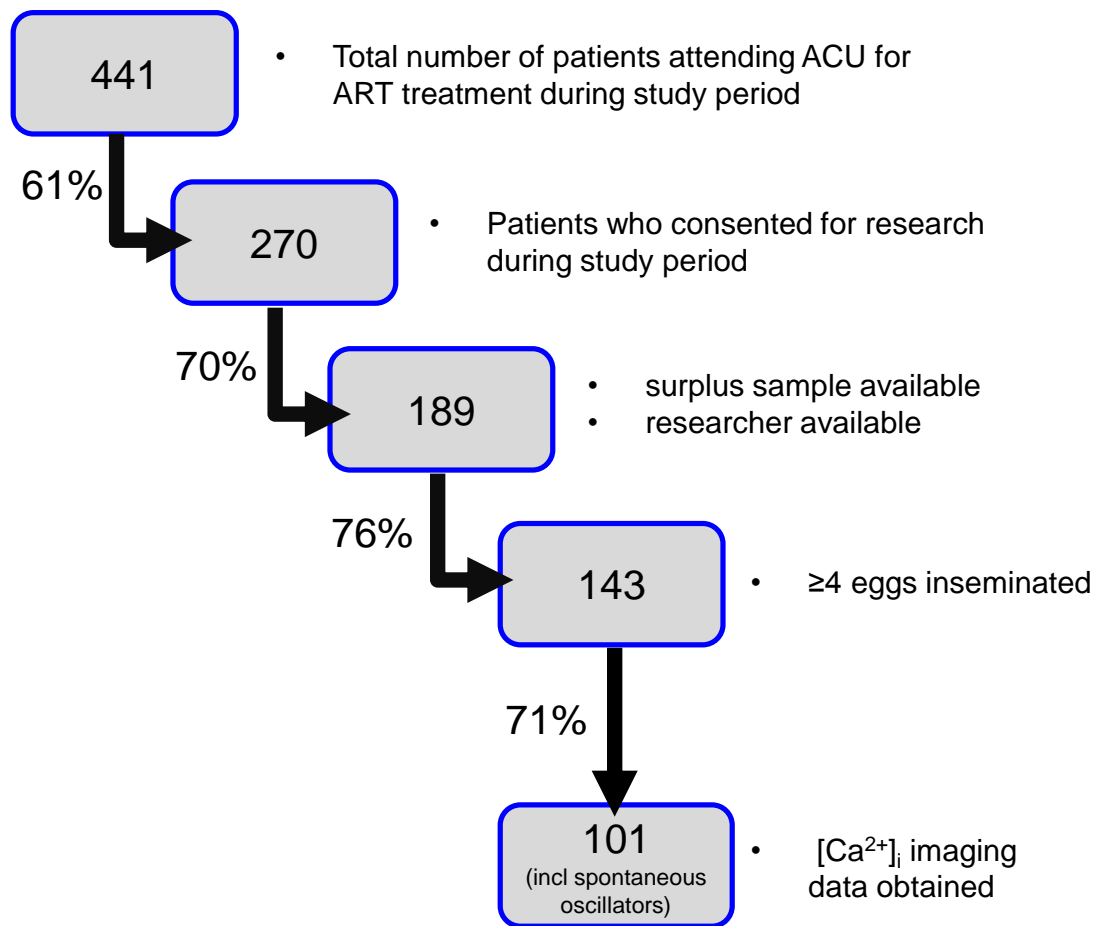
Suppl fig 5



## Suppl fig 6



## Suppl fig 7



final total for study inclusion