Emergence of Smooth Muscle Cell Endothelin B–Mediated Vasoconstriction in Lambs With Experimental Congenital Heart Disease and Increased Pulmonary Blood Flow

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Background—Endothelin-1 (ET-1) has been implicated in the pathophysiology of pulmonary hypertension. In 1-month-old lambs with increased pulmonary blood flow, we have demonstrated early alterations in the ET-1 cascade. The objective of this study was to investigate the role of potential later alterations of the ET cascade in the pathophysiology of pulmonary hypertension secondary to increased pulmonary blood flow.

Methods and Results—Eighteen fetal lambs underwent in utero placement of an aortopulmonary vascular graft (shunt) and were studied 8 weeks after spontaneous delivery. Compared with age-matched control lambs, lung tissue ET-1 levels were increased in shunt lambs (317.2±113.8 versus 209.8±61.8 pg/g, P<0.05). In shunt lambs (n=9), exogenous ET-1 induced potent pulmonary vasoconstriction, which was blocked by the ET A receptor antagonist PD 156707 (n=3). This pulmonary vasoconstriction was mimicked by exogenous Ala 1,3,11,15 ET-1 (4 Ala ET-1), the ET B receptor agonist, and was blocked by the ET B receptor antagonist BQ 788 (n=3). However, in control lambs (n=7), ET-1 and 4 Ala ET-1 did not change pulmonary vascular tone. In contrast to 4-week-old shunt lambs, immunohistochemistry revealed the emergence of ET B receptors on smooth muscle cells in the vasculature of 8-week-old shunt lambs.

Conclusions—Over time, increased pulmonary blood flow and/or pressure results in the emergence of ET B–mediated vasoconstriction, which coincides with the emergence of ET B receptors on smooth muscle cells. These data suggest an important role for ET B receptors in the pathophysiology of pulmonary hypertension in this animal model of increased pulmonary blood flow. (Circulation. 2003;108:1646-1654.)

Key Words: endothelin • pulmonary heart disease • heart defects, congenital
**ET<sub>B</sub> Receptors in Pulmonary Hypertension**

Twenty-three lambs (12 shunted and 11 age-matched controls; Pozzi Livestock, Valley Ford, Calif) then underwent a hemodynamic study as described below. After the last protocol, the lambs were killed by an intravenous injection of pentobarbital sodium (Enthanasia CII, Central City Medical), followed by bilateral thoracotomy. Twelve of the lambs (6 shunted and 6 twin controls) were killed without undergoing any hemodynamic study. These lungs were removed and prepared for tissue ET-1 levels and Western blot analysis, RNase protection assays, and immunohistochemistry.

All protocols and procedures were approved by the Committee on Animal Research of the University of California, San Francisco. All animals were euthanized by appropriate methods as described in the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

**Experimental Protocols**

**In Vivo ET-1–Dependent Responses**

The responses to ET-1 and 4 Ala ET-1 were determined in control lambs at rest and shunt lambs at rest with the vascular graft open. However, the responses to vasodilating agents may be dependent on the resting tone of the vascular bed studied. Therefore, to ensure that response differences were not tone dependent, the vasodilator responses in control lambs were also studied with increased tone during an intravenous infusion of U46619 (a thromboxane A<sub>2</sub> mimic), and shunted lambs were also studied after the vascular graft was closed, when pulmonary blood flow is similar to that of controls.

**Control lambs:** After a 45-minute recovery period from surgery, baseline measurements of the hemodynamic variables were measured. In 7 lambs, ET-1 (250 ng/kg) or 4 Ala ET-1 (1725 ng/kg) was then injected into the pulmonary artery in random order. To determine the selectivity of the agonists and the contribution of each receptor to basal tone, the responses to ET-1 and 4 Ala ET-1 were studied before and during the infusion of PD 156707 (1.0 mg·kg<sup>-1</sup>·h<sup>-1</sup>; an ET<sub>A</sub> receptor antagonist) and BQ 788 (1.0 µg·kg<sup>-1</sup>·min<sup>-1</sup>; an ET<sub>B</sub> receptor antagonist) in 4 additional lambs. After the lambs had recovered from the last agent, an infusion of U46619 (a thromboxane A<sub>2</sub> mimic) was then begun into the inferior vena cava. After 15 minutes of steady-state pulmonary hypertension, baseline measurements were again obtained, ET-1 and 4 Ala ET-1 were administered, and the hemodynamic variables were measured as described above.

**Shunted lambs:** After a 45-minute recovery period from surgery, baseline measurements of the hemodynamic variables were measured. In 9 shunt lambs, ET-1 and 4 Ala ET-1 were then administered as described above. In 3 additional lambs, the responses to ET-1 and 4 Ala ET-1 were studied before and during the infusion of PD 156707 and BQ 788 as described above. The vascular graft was then closed. After a 60-minute recovery, the responses to ET-1 and 4 Ala ET-1 were repeated.

**ET-1 Determinations**

Lung tissues were homogenized in 1 mol/L acetic acid containing 10 µg/mL pepstatin (Peptide International) and immediately boiled for 10 minutes. The homogenates were centrifuged at 25 000 g, and the supernatant was analyzed for immunoreactive endothelin as previously described.

**Tissue Preparation**

The heart and lungs were removed en bloc. The lungs were dissected with care to preserve the integrity of the pulmonary endothelium. Sections (2 to 3 g) from each lobe of the lung were removed. These tissues were snap-frozen in liquid N<sub>2</sub> and stored at −70°C until analysis.

For RNA and protein isolation, the snap-frozen lung tissue was prepared as previously described. For immunohistochemistry, the pulmonary vascular tree was rinsed with cold (4°C) PBS to remove blood and fixed by perfusion with cold (5°C) 4% paraformaldehyde. The pulmonary artery was then clamped. The airways were fixed at 20 cm H<sub>2</sub>O pressure by filling the trachea with cold (4°C) 4%
paraformaldehyde. When the lungs were distended at this pressure, the trachea was clamped. The lungs were fixed for 24 hours at 4°C by immersion in 4% paraformaldehyde. Representative slices from each lobe were removed, placed in 30% sucrose until they sank, placed in OCT, frozen on dry ice, and stored at −70°C until sectioned. Sections (5 to 10 μm) were cut with a cryostat, transferred to aminoalkylsilane-treated slides (Superfrost Plus, Fisher Scientific), and stored at −70°C.16

Western Blot Analysis
Western blot analysis was performed as previously described.14,17 The ETα receptor antiserum was generated as previously described.14 The ETβ receptor antiserum was obtained from Maine Biotechnology Services. The prepro-ET-1 antibody was obtained from Affinity Bioreagents. The specificity of the prepro-ET-1 antibody was verified with a preincubation step with purified ET-1 (50 ng ET-1/15 μL of antiserum) protein. The purified ET-1 was purchased from Sigma. ECE-1α antiserum was generated as previously described.16

Positive controls were run to demonstrate antibody specificity. The methodology and exposure times used were those that we have previously demonstrated to be within the linear range of the autoradiographic film and able to detect changes in lung protein expression.

To compare changes in ETα receptor protein levels between 4 and 8 weeks, protein levels from an additional 4-week-old control lambs and five 4-week-old shunt lambs were analyzed simultaneously with those of 8-week-old lambs.

RNA Probe Synthesis and RNAse Protection Assay
The plasmid containing the cDNA fragment of interest was linearized with the appropriate restriction enzyme (Gibco-BRL). Antisense [32P]UTP radiolabeled cRNA probes (New England Nuclear) were synthesized by in vitro transcription using either T3 or T7 RNA polymerases (Boehringer-Mannheim) in the presence of cold rCTP, rGTP, and rATP.17

RNAse protection assays were performed as previously described.17 Also included was a probe for 18S to serve as a control for comparisons. Comparisons were made by the paired t test using the Bonferroni correction, the unpaired t test, or ANOVA for repeated measures with multiple-comparison testing.

Quantification of autoradiographic results was performed by scanning the bands of interest into an image editing software program (Adobe Photoshop, Adobe Systems). For RNAse protection assays, to control for the amount of input RNA and the recovery of protected probe fragments, the mRNA signal of interest was normalized to the corresponding 18S signal for each lane. The mean±SD was calculated for the relative RNA and protein. The unpaired t test or ANOVA was used for comparisons between 4- and 8-week-old, control and shunt lambs for repeated measures with multiple-comparison testing. A value of P<0.05 was considered statistically significant.

Immunohistochemistry
Immunohistochemistry was performed as previously described.16 Studies were done on serial sections of control and shunt ovine lung with our rabbit anti-ETα antibody raised as described previously.14 In addition, immunohistochemistry was performed with a specific rabbit antiserum raised against ETβ. This antiserum was prepared by injecting rabbits with a highly antigenic protein fragment based on rat ETβ (sequence: NH2-SCLFKFANDHGYNFD-COOH). Rabbis were bled at 6, 8, and 10 weeks, and the 8-week bleed was immunopurified (BioSynthesis Inc). Frozen tissue sections (7 μm) were allowed to come to room temperature. Samples were fixed for 10 minutes in cold acetic acid, then washed 3 times with PBS. To eliminate nonspecific binding of the primary antiserum to tissue proteins, tissue sections were incubated with 1% horse serum in PBS (blocking solution) for 1 hour. Then, tissue sections were incubated with anti-ETα (1:100) or anti-ETβ (1:100), both in the presence of monoclonal smooth muscle cell–actin antibody (1:400, Sigma) both in blocking solution at 4°C overnight. After 3 washes with PBS for 5 minutes, samples were hybridized with Rhodamine Red-X goat anti-rabbit (to stain ET receptors) and Oregon Green 488 goat anti-mouse secondary antibodies (to stain smooth muscle cell–actin) (Molecular Probes) at a concentration of 1:400 in blocking solution for 45 minutes at room temperature. After 3 further washes with PBS, an antifading solution was added, and samples were visualized by confocal microscopy. A minimum of 3 different sets of control and shunt lung tissue were prepared and examined.

Lung sections from four 4-week-old shunt and control lambs were also analyzed to localize ETα and ETβ receptors.

Results
At 2 months of age, shunted lambs weighed significantly less than controls (18.9±4.9 versus 22.6±4.2 kg, P<0.05). The ratio of pulmonary to systemic blood flow (Qp/Qs) was 3.0±0.9. Mean pulmonary arterial pressure was increased to 41% of systemic values. This was associated with an increase in pulmonary blood flow and left atrial pressure (P<0.05). Mean systemic arterial pressure and the calculated left pulmonary vascular resistance were decreased (P<0.05) (Table). Peripheral lung tissue immunoreactive ET-1 concentrations were greater in shunted lambs than control lambs (317.2±113.8 versus 209.8±61.8 pg/g, P<0.05).

The baseline hemodynamics and protein concentrations of the 4-week-old lambs used in this study were similar to those previously published (data not shown).15,14 In addition, the
baseline hemodynamics of the 4- and 8-week-old shunt lambs used in this study were similar.

**Hemodynamic Study**

**Control Lambs**

In control lambs, the intrapulmonary injection of ET-1 and 4 Ala ET-1 did not change mean pulmonary arterial pressure, left pulmonary blood flow, or left pulmonary vascular resistance (Figure 1). ET-1 increased mean systemic arterial pressure from 67.5/110.0 to 90.6/110.8 mm Hg (P < 0.05). Similarly, during steady-state pulmonary hypertension induced by the infusion of U46619, intrapulmonary injections of ET-1 or 4 Ala ET-1 did not change mean pulmonary arterial pressure or pulmonary vascular resistance (data not shown).

At rest, the infusion of PD 156707 induced a modest decrease in mean pulmonary arterial pressure (−8.0 ± 0.6%, P < 0.05) and left pulmonary vascular resistance (−9.9 ± 2.6%, P < 0.05) (n=4). Pulmonary blood flow was unchanged. During the infusion of PD 156707, the increase in systemic arterial pressure induced by ET-1 was blocked, and the response to 4 Ala ET-1 was unchanged (data not shown). The infusion of BQ 788 did not change the baseline hemodynamic variables (n=4). In addition, during the infusion of BQ 788, the response to ET-1 and 4 Ala ET-1 was unchanged (data not shown).

**Shunted Lambs**

In shunted lambs with the vascular graft open, the intrapulmonary injection of ET-1 increased mean pulmonary arterial pressure (25.1 ± 8.3 to 35.2 ± 11.8 mm Hg) and systemic (63.7 ± 12.2 to 73.3 ± 14.2) arterial pressure and left pulmonary vascular resistance (0.13 ± 0.04 to 0.23 ± 0.11 mm Hg · L⁻¹ · min⁻¹) (P < 0.05). Left pulmonary blood flow was unchanged. Similarly, the intrapulmonary injection of 4 Ala ET-1 increased mean pulmonary arterial pressure (25.0 ± 9.7 to 32.3 ± 12.0 mm Hg) and systemic (66.8 ± 12.4 to 73.6 ± 13.1 mm Hg) arterial pressure and left pulmonary vascular resistance (0.13 ± 0.07 to 0.22 ± 0.16 mm Hg · L⁻¹ · min⁻¹) (P < 0.05). Left pulmonary blood flow decreased (132.7 ± 41.3 to 122.1 ± 43.7 mL/min) (P < 0.05).

Similarly, after shunt closure, the intrapulmonary injections of 4 Ala ET-1 and 4 Ala ET-1 induced similar increases in mean pulmonary arterial pressure and left pulmonary vascular resistance (data not shown) (P < 0.05).

The percent increase in both left pulmonary vascular resistance and mean pulmonary arterial pressure induced by the injection of ET-1 was greater in shunted lambs than in control lambs (P < 0.05) (Figure 1). Similarly, the percent increase in both left pulmonary vascular resistance and mean pulmonary arterial pressure induced by the injection of 4 Ala ET-1 was greater in shunted lambs than in control lambs (P < 0.05) (Figure 1).

The infusion of PD 156707 decreased mean pulmonary arterial pressure (−15.4 ± 3.7%, P < 0.05) and left pulmonary vascular resistance (−25.0 ± 3.9%, P < 0.05) (n=3). Pulmonary blood flow was unchanged. During the infusion of PD 156707, the increase in left pulmonary vascular resistance induced by ET-1 was blocked (90.1 ± 35.2% versus
7.9±18.5%), but the response to 4 Ala ET-1 was unchanged. The infusion of BQ 788 did not change the baseline hemodynamic variables. However, during the infusion of BQ 788, the increase in left pulmonary vascular resistance in response to ET-1 was partially attenuated (90.1±35.2% versus 19.2±16.5%), whereas the response to 4 Ala ET-1 was completely blocked (107.5±22.0% versus 1.9±5.7%) (n=3).

To determine whether the alterations in ET-1 levels and physiological responses were associated with changes in gene expression, we performed Western blot analysis and RNAse protection assays. Compared with age-matched control lambs, the levels of both prepro-ET-1 and ECE-1 protein and mRNA were unchanged (Figures 2 and 5). Protein levels of ETA receptors were increased in shunt lambs (P<0.05), whereas the changes in ET$_A$ receptor mRNA did not reach statistical significance (Figures 3 and 5). Protein and mRNA levels of ET$_B$ receptors were also unchanged in shunt lambs compared with age-matched controls (Figures 3 and 5). However, compared with 4-week-old shunt lambs, protein levels of ET$_B$ receptor were significantly increased in 8-week-old shunt lambs (Figure 4).

In both 4- and 8-week-old control lambs, localization by immunohistochemistry demonstrated ET$_B$ receptors solely on vascular endothelial cells (Figure 6C and 6G) and ET$_A$ receptors solely in the vascular smooth muscle cell layer (Figure 6A and 6E). In 4- and 8-week-old shunt lambs ET$_A$ receptors were again found solely in the vascular smooth muscle cell layer (Figure 6B and 6F). In 4-week-old shunt lambs, ET$_B$ receptors were found predominantly on endothelial cells (>90%) and occasionally on smooth muscle cells. However, in 8-week-old shunt lambs, a significant subpopulation of ET$_B$ receptors were localized to the vascular smooth muscle cell layer (>50%) (Figure 6D and 6H).

**Discussion**

In a follow-up investigation to our previous studies, we found that exposure to 8 weeks of increased pulmonary blood flow and pressure results in progressive alterations in ET-1 signaling. Similar to alterations displayed at 4 weeks, shunted lambs had increased lung tissue concentrations of ET-1 and increased protein levels of ET$_A$ receptors. However, in contrast to decreased ET$_B$ protein levels and a loss of ET$_B$ receptor-mediated pulmonary vasodilation in 4-week-old shunt lambs, 8-week-old shunt lambs had an upregulation of ET$_B$ receptor protein and the emergence of ET$_B$ receptor-mediated pulmonary vasoconstriction. Immunohistochemistry revealed that both 4- and 8-week-old control lambs had ET$_B$ receptors solely on endothelial cells, whereas 8-week-old shunt lambs displayed a subpopulation of ET$_B$ receptors present in the vascular smooth muscle cell layer. ET$_A$ receptors were found on vascular smooth muscle cells in all groups. These data suggest a potentially important role for...
smooth muscle cell ET_b receptors in the pathophysiology of pulmonary hypertension secondary to congenital heart disease.

The hemodynamic effects of ET-1 are mediated by at least 2 populations of receptors, ETA and ET_b receptors. In the systemic vasculature, the response to exogenous ET-1 is consistently vasoconstricting, which is mediated via ETA receptor activation. However, in the pulmonary vasculature, the response to exogenous ET-1 is dependent on a variety of factors, including age, species, and the underlying disease state. In the fetal and young sheep, ET-1 induces pulmonary vasodilation, which is mediated via ET_b receptor activation and the release of NO. In the adult sheep, ET-1 induces pulmonary vasoconstriction, which is associated with prostaglandin release and presumed ETA receptor activation. In the rabbit, rat, and human adult pulmonary circulation, pulmonary vasoconstricting ET_b receptors have been identified in the medial layer of pulmonary arteries. However, in response to modest doses of ET-1, ET_b receptor–induced pulmonary vasoconstriction has not been reported previously in the young sheep or human. With the use of 4 Ala ET-1, a selective ET_b receptor agonist, we demonstrate significant pulmonary vasoconstriction in 8-week-old shunt lambs and no response in age-matched control lambs. In addition, the infusion of BQ 788 (an ET_b receptor antagonist) blocked the vasoconstriction induced by 4 Ala ET-1, whereas PD 156707 (an ETA receptor antagonist) did not, further suggesting that 4 Ala ET-1–induced pulmonary constriction was secondary to ET_b receptor activation. Interestingly, the infusion of BQ 788 did not change resting pulmonary vascular tone in either control or shunt lambs, suggesting that ET_b receptor activation participates in agonist-induced responses but makes a minimal contribution to basal tone. Conversely, the increase in ET_b receptor protein noted in shunt lambs was associated with an augmented pulmonary vasodilating response to PD 156707, the ETA receptor antagonist, suggesting that ETA receptor activation contributes to basal tone in shunt lambs.

In association with the emergence of ET_b receptor–mediated pulmonary vasoconstriction, there were changes in protein levels of ET_b receptors. Previously, in 4-week-old shunt lambs, we demonstrated a decrease in ET_b receptor lung protein levels compared with age-matched control lambs. This was associated with loss of the pulmonary vasodilating response to the ET_b receptor agonist 4 Ala ET-1. In the present study, we demonstrate no difference in lung ET_b receptor protein between 8-week-old shunt lambs and age-matched control lambs. However, compared with 4-week-old shunt lambs, there was a significant increase in ET_b receptor protein levels. In addition, immunohistochemistry reveals that a subset of ET_b receptor expression predominantly in 8-week-old shunt lambs now localizes to the smooth muscle cell layer. Taken together, these data suggest that increased pulmonary blood flow and/or pressure causes a decrease in endothelial ET_b receptors and the emergence of smooth muscle cell ET_b receptors. Because 4 Ala ET-1–induced pulmonary vasoconstriction is now present in 8-week-old shunt lambs, we speculate that the increased protein levels of ET_b receptor at 8 weeks mediate the demonstrated pulmonary vasoconstricting response. Although increasing in vitro data suggest that changes in mechanical forces alter ET-1 signaling, few data are available on the regulation of ET receptor signaling. Therefore, the potential mechanisms for the current receptor changes are unknown and warrant further investigation.

Changes in ET_b receptors have previously been demonstrated in other animal models of pulmonary hypertension. For example, in contrast to the present study, rats exposed to monocrotaline displayed an increase in ET_b receptor–mediated vasodilation during the development of pulmonary hypertension. In addition, in piglets exposed to hypoxia, there was loss of the normal postnatal increase in endothelial ET_b receptors during the first 3 days of life, and in sheep with persistent pulmonary hypertension of the newborn, ET_b receptor expression was decreased. However, in humans with advanced pulmonary vascular disease secondary to thromboembolic pulmonary hypertension, a recent report demonstrates a selective upregulation of ET_b mRNA in the media of pulmonary arteries. The present study represents the first investigation of changes in ET receptors during the development of pulmonary hypertension secondary to congenital heart disease with increased pulmonary blood flow. Our findings are consistent with the recent findings in adults and further suggest an important role.
for both ET$_A$ and ET$_B$ receptors in the pathophysiology of pulmonary hypertensive disorders.

In summary, in a model of increased pulmonary blood flow and pulmonary hypertension secondary to in utero aortopulmonary vascular graft placement, the present study describes the emergence of smooth muscle cell, ET$_B$ receptor–mediated pulmonary vasoconstriction over the second month of postnatal life. In addition, there are persistent increases in lung tissue ET-1.

Figure 5. RNAase protection assays for prepro-ET-1, ECE-1, ET$_A$ receptor, and ET$_B$ receptor. A, cRNA probe for ovine prepro-ET-1 was hybridized overnight to 50 µg of total lung RNA prepared from two 8-week-old lambs (1 control and 1 shunt). Densitometric determinations from 5 control and 5 shunt lambs did not reveal differences (data not shown). B, cRNA probe for ovine ECE-1 was hybridized overnight to 50 µg of total lung RNA prepared from two 8-week-old lambs (1 control and 1 shunt). Densitometric determinations from 5 control and 5 shunt lambs did not reveal differences (data not shown). C, cRNA probe for ovine ET$_A$ receptor was hybridized overnight to 50 µg of total lung RNA prepared from two 8-week-old lambs (1 control and 1 shunt). Densitometric determinations from 5 control and 5 shunt lambs did not reveal differences (data not shown). D, cRNA probe for ovine ET$_B$ receptor was hybridized overnight to 50 µg of total lung RNA prepared from two 8-week-old lambs (1 control and 1 shunt). Densitometric determinations from 5 control and 5 shunt lambs did not reveal differences (data not shown). No protected fragments were detected in lanes in which probe was hybridized without RNA (P$_N$) or in presence of tRNA. A cRNA for ovine 18S was also hybridized to serve as a control for RNA loading.
levels and ET\textsubscript{A} receptor protein levels. These data suggest an increasingly important role for both ET\textsubscript{A} and ET\textsubscript{B} receptors in the pathophysiology of pulmonary hypertension secondary to congenital heart defects. With the recent development of ET receptor antagonists as potential therapeutic agents, these data have important clinical implications. Similar to the recent report in adults with advanced disease, these data suggest that combined ET\textsubscript{A} and ET\textsubscript{B} receptor antagonists may be more beneficial than selective ET\textsubscript{A} receptor antagonists in the treatment of pulmonary hypertension. In fact, bosentan, a combined receptor antagonist, has recently shown beneficial therapeutic effect in adults with primary pulmonary hypertension. Further investigations into the mechanisms of these alterations are needed and may have important clinical implications.

Figure 6. ET\textsubscript{A} and ET\textsubscript{B} protein expression in lung in vivo from tissue from 4- and 8-week-old lambs. Immunohistochemical localization of ET\textsubscript{A} and ET\textsubscript{B} protein expression in lung in vivo from 4- (A–D) and 8-week-old (E–H) lambs. Polyclonal rabbit ET\textsubscript{A} and ET\textsubscript{B} receptor antibodies and monoclonal mouse anti-smooth muscle cell (SMC)–actin antibodies were used to localize expression. ET\textsubscript{A} (A, B, E, F) and ET\textsubscript{B} (C, D, G, H) protein expression is shown in red and SMC-actin expression in green. Colocalization is shown in yellow. Magnification \( \times 800 \). In both shunt and control lambs, ET\textsubscript{A} receptors localize to smooth muscle cells at both ages. In 4- and 8-week-old control lambs (A, C, E, G), ET\textsubscript{B} receptors localize to endothelial cells. In 4-week-old shunt lambs, ET\textsubscript{B} receptors were found predominantly on endothelial cells (>90%) (D). However, in the majority of 8-week-old shunt lamb vessels (>50%), subpopulations of ET\textsubscript{B} receptors localize to smooth muscle cells (H). Results are representative of 3 different sets of twin matches (control and shunt).
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